Reproductive management and gamete quality in pikeperch (*Sander lucioperca*)

Dissertation

zur Erlangung des akademischen Grades

doctor rerum agriculturarum
(Dr. rer. agr.)

eingereicht an der Lebenswissenschaftlichen Fakultät
der Humboldt-Universität zu Berlin

von

M. Sc. Fabian Johannes Schäfer

Präsidentin der Humboldt-Universität zu Berlin: Prof. Dr.-Ing. Dr. Sabine Kunst

Dekan der Lebenswissenschaftlichen Fakultät: Prof. Dr. Bernhard Grimm

Gutachter: 1. Prof. Dr. Werner Kloas

2. PD Dr. Klaus Knopf

Tag der mündlichen Prüfung: 09.11.2016

This thesis was conducted at the

Leibniz-Institute of Freshwater Ecology and Inland Fisheries (IGB)

Department of Ecophysiology and Aquaculture

Berlin, Germany

Fabian Johannes Schäfer

Reproductive management and gamete quality in pikeperch (*Sander lucioperca*)

Dissertation

Humboldt-Universität zu Berlin, Germany (2016)

Acknowledgements

Nobody said that pursuing a doctoral thesis is going to be an easy task. However, what I experienced during the last few years is that having great and supportive people around you can make a lot of things easier. First, I would like to express my gratitude to my supervisors Werner Kloas and Sven Würtz for providing the opportunity to work on this exciting topic, as well as for their guidance and support throughout my time at the IGB. Special thanks go to Sven Würtz, whose expertise, advice and dedication played an essential role in this project and in the development of my scientific career. Also, I would like to thank all cooperation partners, especially Julia Overton and the staff of AquaPri, who have hosted me and provided valuable advice whenever needed, as well as precious sampling material, which made this project possible. Engaging with scientists and farmers within the percid aquaculture community was a great experience, especially with people like Stefan Meyer, whose devotion and positive conviction were a great source of motivation. I am equally grateful for the support of my friends and colleagues at the IGB, particularly members of the department V and the working group Applied Physiology and Sustainable Aquaculture, as well as the technical staff and people involved in the doctoral program. Finally, my very sincere thanks go to my family and friends, especially my wonderful girlfriend Sarah, for their emotional support during the entire time.

Contents

Summary

Pikeperch (*Sander lucioperca*) is among the species with highest potential for European aquaculture diversification. Despite the development of commercially applied rearing and reproduction protocols, the propagation of pikeperch is still impeded by a shortage of stocking material caused by low fertilization success and high embryo mortality. The main aims of this thesis were (I) to identify potential pitfalls for gamete quality, such as year-round reproduction or currently applied fertilization protocols, (II) to further understand the interactions between maternal characteristics and egg quality parameters and how these properties affect the developmental potential of the oocytes and (III) to evaluate predictive biomarkers of future egg development under commercial hatchery conditions.

In contrast to the hypothesized adverse effects on reproductive success, no substantial consequences of year-round, out-of-season reproduction on gamete quality could be detected. In addition, rates of fertilization, embryo survival and hatching were on average relatively high confirming the effectiveness of the applied reproduction protocols. It was possible to attribute variability in fecundity and egg quality to maternal characteristics, especially female length. A female length of ~65 to 70 cm could be identified, which was associated with high reproductive performance. Additionally, there were indications regarding maternal processes, such as handling induced, as well as oxidative stress, spawning history and time, which affected the composition of the oocytes. In turn, it was possible to identify specific egg quality parameters, which were correlated with the developmental potential of the oocytes from fertilization until hatching. These predictive biomarkers include egg diameter, specific fatty acids (FA), polar 15:0, 18:0, 20:5(n-3) and the ratio of neutral 22:6(n-3) to 20:5(n-3), as well as dry weight content. In contrast, other egg parameters (cortisol levels, mtDNA fragmentation, antioxidant capacity, maternal mRNA of probibitin2) could not be used to characterize egg quality and to directly predict future embryo development suggesting potent coping mechanisms. However, strong interactions between specific egg components, especially between FA and cortisol content, as well as markers of oxidative stress, were observed highlighting the importance of physiological process involved in shaping egg

properties. In contrast, mtDNA damage was independent of antioxidant capacity. Generally, FA could be identified as integrative parameter linking oocyte composition to physiological processes during reproduction, which affected egg quality.

These findings highlight the strong interactions of maternal characteristics and egg properties. Consequently, a substantial variability in egg developmental performance could be explained (47.1% fertilization; 58.2% 24 h, 47% 48 h, 43.9% 72 h embryo survival; 46.6% hatching; 88.9% hatched larvae) by a combination of maternal traits and egg quality parameters (egg size, polar 20:5(n-3), 15:0 and 18:0 FA, fecundity, female length). The inherent biochemical oocyte properties were critical especially during fertilization and early embryogenesis (until 48 h post-fertilization) when variability and mortality were highest. Still, it remains speculative whether specific observations, e.g., the relation of mtDNA damage and FA content, were a result of oocyte based physiology, e.g., mitochondria dysfunction, or if they indicate suboptimal conditions or even adaptive strategies on the maternal level.

Despite the overall high fertilization observed here (89.2% on average), there is still potential for fine tuning of protocols for *in vitro* fertilization in pikeperch with regard to the management of male gametes. Sperm of different males is often pooled prior to fertilization or stored for short periods (hours) until ovulated eggs become available. A loss of sperm quality (motility, velocity) could not be prevented during short-term storage using sperm extenders and enhancement supplements (melatonin, progesterone). A novel approach was applied to assess pooling effects by cross-wise transfusion of sperm and seminal fluid (SF) of males with differing initial sperm quality. Transfusion of SF between low and high quality sperm resulted in a significant decrease in sperm with high initial velocity, whereas the velocity of low quality sperm could not be improved. Conclusively, pooling sperm of males with different quality, as well as short-term storage has a significant adverse effect on sperm performance. Pooling should only be considered if the sperm quality is known.

The findings of this thesis deliver valuable management advice for hatcheries and indicate potential for optimization in regard to *in vitro* reproduction protocols in pikeperch. Furthermore, these results allow for conclusions in respect of physiological processes, which are crucial for determining the developmental potential of oocytes. Clearly, a multi-layered, holistic approach is beneficial when studying gamete quality in fish.

Zusammenfassung

Der Zander (*Sander lucioperca*) ist eine der Arten mit dem größten Potential für die Diversifizierung der Aquakultur in Europa. Trotz der Entwicklung von kommerziell genutzten Haltungs- und Vermehrungsprotokollen wird die Verbreitung des Zanders durch einen Mangel an Satzmaterial erschwert, welcher durch einen geringen Befruchtungserfolg und hohe Embryosterblichkeit hervorgerufen wird. Die Hauptziele dieser Dissertation waren (I) mögliche Ursachen für die Schwankungen der Qualität der Gameten, wie ganzjährige Reproduktion oder derzeit verwendete Befruchtungsprotokolle, zu identifizieren, (II) die Interaktionen zwischen maternalen Merkmalen und Eiqualitätsparametern besser zu verstehen und wie diese Eigenschaften das Entwicklungspotential der Oozyten beeinflussen und (III) prädiktive Biomarker der Eientwicklung unter kommerziellen Bedingungen in Brutbetrieben zu evaluieren.

Entgegen der Annahme, dass sich die ganzjährige Reproduktion außerhalb der natürlichen Laichsaison negativ auf den Reproduktionserfolg auswirkt, konnten keine wesentlichen Konsequenzen für die Qualität der Gameten identifiziert werden. Zusätzlich waren die Befruchtungs-, Embryoüberlebens- und Schlupfraten durchschnittlich relativ hoch. Dies unterstreicht die Effektivität der verwendeten Reproduktionsprotokolle. Es war möglich Schwankungen in der Fekundität und der Eiqualität maternalen Merkmalen zuzuordnen, besonders der Größe des Weibchens. Eine Länge der Weibchen von ~65 bis 70 cm war mit hohem Reproduktionserfolg assoziiert. Zusätzlich gab es Anzeichen im Bezug auf maternale Prozesse, wie Handling oder oxidativen Stress, Laicherfahrung und den Zeitpunkt der Eiablage, welche die Zusammensetzung der Oozyten beeinflusste. Es war wiederum möglich, spezifische Eiqualitätsparameter zu identifizieren, die mit dem Entwicklungspotential der Eier von der Befruchtung bis zum Schlupf korreliert waren. Diese prädiktiven Biomarker umfassen Eidurchmesser, bestimmte polare Fettsäuren, 15:0, 18:0 und 20:5(n-3), das Verhältnis der neutralen Fettsäuren 22:6(n-3) zu 20:5(n-3), sowie der Anteil des Trockengewichts. Andere Eiparameter (Cortisolgehalt, mtDNA Fragmentierung, antioxidative Kapazität, maternale mRNA von Prohibitin2) konnten jedoch nicht zur Charakterisierung der

Eiqualität oder zur Vorhersage der Embryonalentwicklung verwendet werden. Dies spricht für die Existenz wirkungsvoller Bewältigungsstrategien. Es wurden allerdings starke Zusammenhänge zwischen bestimmten Eibestandteilen beobachtet, besonders zwischen Fettsäuren und Cortisol, sowie Markern für oxidativen Stress. Dies unterstreicht die Bedeutung physiologischer Prozesse während der Prägung der Eigenschaften der Eier. Demgegenüber waren Schäden der mtDNA von der antioxidativen Kapazität unabhängig. Fettsäuren konnten generell als integrative Parameter identifiziert werden, welche die Zusammensetzung der Oozyten mit physiologischen Prozessen während der Reproduktion verbinden, was wiederum die Eiqualität beeinflusst.

Diese Ergebnisse unterstreichen die starken Interaktionen zwischen maternalen Merkmalen und den Eigenschaften der Eier. Folglich war es möglich einen erheblichen Anteil der Variabilität in der Ei- bzw. Embryonalentwicklung (47.1% Befruchtung; 58.2% 24 h, 47% 48 h, 43.9% 72 h Embryoüberleben; 46.6% Schlupf; 88.9% geschlüpfte Larven) durch eine Kombination von maternalen Merkmalen und Eiqualitätsparametern (Eidurchmesser, polare Fettsäuren 20:5(n-3), 15:0 und 18:0, Fekundität, Länge des Weibchens) zu erklären. Die inhärente biochemische Zusammensetzung der Oozyten war besonders während der Befruchtung und der frühen Embryogenese (bis 48 h nach dem Schlupf) von großer Bedeutung. In diesem Zeitraum waren die Variabilität und die Sterblichkeit besonders hoch. Es bleibt jedoch offen, ob bestimmte Beobachtungen, wie z. B. der Zusammenhang von Schäden der mtDNA und dem Fettsäuregehalt, durch physiologische Prozesse innerhalb der Oozyten, z. B. Fehlfunktion der Mitochondrien, oder durch suboptimale maternale Bedingungen oder adaptive Strategien hervorgerufen werden.

Obwohl insgesamt ein hoher Befruchtungserfolg (durchschnittlich 89.2%) verzeichnet wurde, gibt es Möglichkeiten um die Feinabstimmung von Protokollen für die *in vitro* Befruchtung hinsichtlich der Handhabung männlicher Gameten beim Zander zu optimieren. Die Spermien verschiedener Männchen werden häufig vor der Befruchtung zusammengeführt (Pooling) oder für einen kurzen Zeitraum (Stunden) gelagert bis ovulierte Eier zur Verfügung stehen. Ein Verlust der Spermienqualität (Motilität, Geschwindigkeit) während kurzzeitiger Lagerung konnte durch die Verwendung von Spermienextendern oder die Zugabe von Ergänzungsmitteln (Melatonin, Progesteron) nicht verhindert werden. Um Poolingeffekte zu analysieren wurde eine neuartige Methode angewandt, bei der Spermien und Seminalflüssigkeit von Männchen mit anfänglich unterschiedlicher Spermienqualität

kreuzweise gemischt wurden. Die Transfusion von Seminalflüssigkeit zwischen Spermien mit niedriger und hoher Qualität resultierte in einer signifikanten Abnahme bei Männchen mit anfänglich hoher Spermiengeschwindigkeit, während die Geschwindigkeit von Spermien mit niedriger Qualität nicht gesteigert werden konnte. Es ist abschließend festzuhalten, dass das Pooling der Spermien von Männchen mit unterschiedlicher Qualität, als auch die kurzzeitige Lagerung signifikant negative Auswirkungen auf die Eigenschaften der Spermien haben. Pooling sollte dementsprechend nur bei bekannter Spermienqualität angewandt werden.

Die Erkenntnisse dieser Dissertation liefern wertvolle Empfehlungen für das Management von Brutbetrieben und zeigen Potential zur Optimierung von Reproduktionsprotokollen bezüglich der *in vitro* Befruchtung beim Zander auf. Die Ergebnisse ermöglichen außerdem Rückschlüsse auf physiologische Prozesse, die das Entwicklungspotential der Oozyten maßgeblich beeinflussen. Es konnte deutlich gezeigt werden, dass ein vielschichtiger, holistischer Ansatz bei der Untersuchung der Gametenqualität bei Fischen von Vorteil ist.

List of Tables

List of Figures

General introduction

1 Aquaculture: Current status and the need for diversification

In contrast to the stagnating capture fisheries, the global aquaculture production is rapidly increasing reaching 97.2 million tons (live weight) with an estimated value of 157 billion USD in the year 2013, exceeding the fisheries landings (FAO, 2015). Regarding finfish, 43.1% of the global production was supplied by aquaculture in 2013 (total of 41.3 million tons) compared to 30.6% in the year 2003 and the inland finfish production is contributing disproportionally (64.9%) to this growth (FAO, 2015). Still, global production and consumption is not distributed equally and the European Union depends on imports indicating the need for expansion of local production (FAO, 2014). In addition, ~85% of the global production of finfish comes from only 15 species (Teletchea and Fontaine, 2014) resulting in vulnerability of this sector towards the spread of diseases and parasites, such as the koi herpes virus in carp species (Hedrick et al., 2000) or sea lice in salmon (*Salmo salar*) (Morton and Routledge, 2005). In addition, the introduction of farmed non-native species into new environments is associated with the risk of escapees, which threaten the local environment. For example, the rapid spread of Asian carp species in North America causes extensive and irreversible changes to the endemic ecosystem (Conover et al., 2007).

In Central Europe, aquaculture of freshwater fish (mainly trout and carp species) is traditionally based on extensive and semi-extensive pond farming. Despite the benefits of low investment and production costs, this technique has disadvantages impeding the potential for up-scaling, such as spatial restrictions and environmental regulations. More importantly, pond-based farming strongly depends on water supply and environmental conditions (e.g., annual fluctuations in temperature), which interferes with optimal growth rates of fish and limits reproduction of temperate species to a single spawning event per year resulting in production peaks with low market prices during the spawning season. During the last decades, a technology-driven approach to aquaculture fueled the development of intensive farming in fully or partly enclosed recirculating aquaculture systems (RAS). This technique aims for the

full control of the environmental conditions applying high stocking densities, allowing a constant, year-round market supply while reducing the environmental impact. However, high investment and operating costs require farming of species with high market acceptance and value. Therefore, the introduction of new, endemic species is a prerequisite for the successful development of (European) inland, freshwater aquaculture.

2 Pikeperch: An emerging species for intensive aquaculture

2.1 Biology of pikeperch

The pikeperch (*Sander lucioperca*) is a piscivorous, nocturnal and twilight-active percid, which occurs mainly in freshwater – to a certain extent also in brackish waters with low salinities – all over Europe and was also introduced to other countries, such as Turkey, Morocco and Tunisia (Fig. 0.1; M'Hetli et al., 2011; Lappalainen et al., 2003; Lehtonen et al., 1996). It can reach sizes of above a meter, but typically has a length of 30 to 70 cm. Pikeperch are ambush-pursuit, pack-hunting predators, depending on their excellent olfactory and optic senses preferring turbid waters (Feiner and Höök, 2015).

Fig. 0.1. Photographic image of a female pikeperch taken by the author.

As in other temperate species, age and size at maturation in pikeperch follow a latitudinal gradient across their geographic distribution (Fontaine et al., 2015; Lappalainen et al., 2003). Males typically mature one year earlier than females at an age of 2 to 3 years (Raikova-Petrova and Zivkov, 1998; Lappalainen et al. 2003). Males and females cannot be distinguished with certainty by morphological characteristics, particularly outside of the spawning season. The neuroendocrine control of reproduction in pikeperch does not principally differ from other temperate fish species and is mainly determined by

environmental cues (Fig. 0.2; Feiner and Höök, 2015; Fontaine et al., 2015; Żarski et al., 2015). In brief, signals, such as temperature or photoperiod, activate the expression of the gonadotropin releasing hormone (GnRH), which induces the liberation of luteinizing (LH) and follicle stimulating hormone (FSH) from the pituitary into the blood stream, which activates specific receptors in the gonads and regulates germ cell development (Taranger et al., 2010; Żarski et al., 2015). Gonadal sex steroids form a feedback loop exerting positive and negative effects on the brain and the pituitary modulating FSH and LH secretion in a stage-specific way (Taranger et al., 2010; Żarski et al., 2015). This pathway is commonly referred to as hypothalamus-pituitary-gonad (HPG) axis.

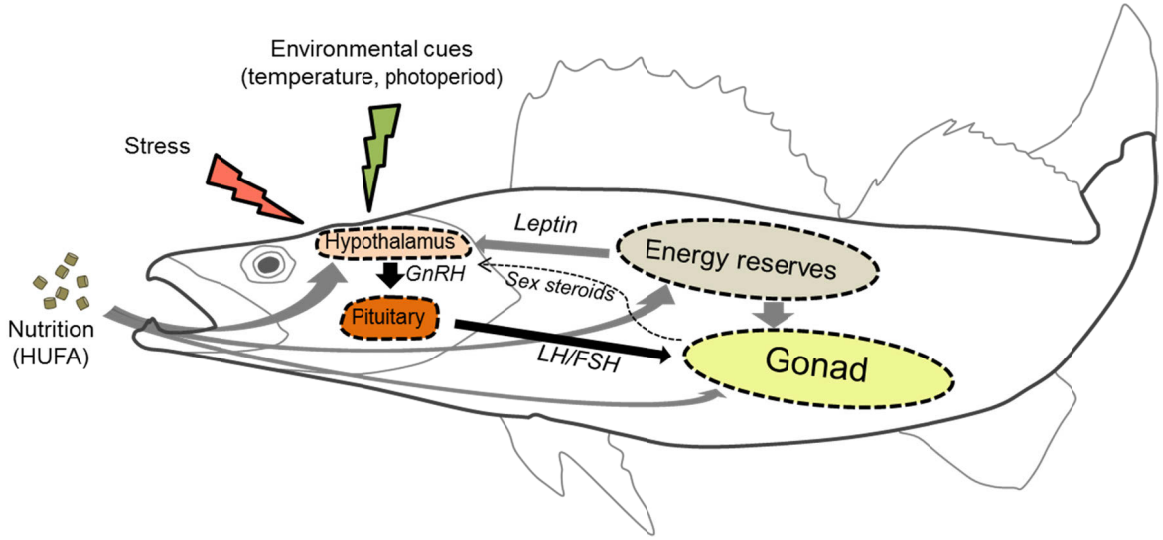


Fig. 0.2. Schematic overview of external and internal parameters acting on the neuroendocrine control of reproduction in pikeperch. Environmental cues and endogenous signals (e.g., leptin) activate secretion of the gonadotropin releasing hormone (GnRH) in the hypothalamus inducing the secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH) in the pituitary. Gonadal sex steroids form a feedback loop to the brain. Stress and nutrition, especially highly unsaturated fatty acids (HUFA), modulate aspects of the endocrine control. Energy reserves are mobilized during gonad maturation.

In addition to environmental cues, other external and internal parameters exert an influence on the neuroendocrine control of reproduction, such as nutrition, energy reserves and stress. Nutrition affects the condition of an individual indirectly triggering an endocrine response, while certain components of the diet, especially highly unsaturated fatty acids (HUFA), are directly involved acting on the brain and/or the gonad (Cerdá et al., 1995; Mercure and Van Der Kraak, 1996). The nutritional state of an individual is linked to the HPG

axis by the actions of the adipocyte derived hormone leptin on the expression of GnRH (Oakley et al., 2009; Taranger et al., 2010). Dietary HUFA on the other hand, are precursors of eicosanoids and are involved in steroidogenesis (Izquierdo et al., 2001). Stress via cortisol has adverse effects on reproduction and can even induce a complete inhibition of reproductive function (Milla et al., 2009; Schreck et al., 2001).

In pikeperch, decreasing temperatures below a threshold and – to a certain extent – day length in autumn activate the HPG axis and nutrients are mobilized and directed to the maturing gonads during the winter (Feiner and Höök, 2015; Fontaine et al., 2015; Żarski et al., 2015). Increasing temperatures and day length during spring induce gonad maturation. Spermiation/ovulation and group-synchronous spawning takes place between April and June when water temperatures reach a threshold of 12 to 15 °C in Central Europe (Feiner and Höök, 2015; Fontaine et al., 2015; Lappalainen et al., 2003). Males build a crater-like nest and darken. After forming pairs, females spawn all eggs at once into the nest, which are fertilized and guarded by the male until hatching of the offspring after 100 to 110 degree days (Schlumberger and Proteau, 1996). Pikeperch are highly fecund with ~200,000 eggs kg^{-1} female body weight, ranging from 50,000 to 450,000 eggs kg^{-1} (Lappalainen et al., 2003; Lehtonen et al. 1996; Steffens et al., 1996). The eggs contain an oil globule, are adhesive and at a size of 0.5 to 1.4 mm they are relatively small compared to eggs of other freshwater percids (Feiner and Höök, 2015; Lappalainen et al., 2003; Schlumberger and Proteau, 1996). Larvae hatch with an average body length of ~5 mm and start external feeding after 100 to 110 degree days (Schlumberger and Proteau, 1991, 1996; Steffens et al., 1996). During the first year, juveniles typically shift from zooplanktivory to piscivory, which fuels fast growth (Ljunggren, 2002; Van Densen et al., 1996).

2.2 Current status and practice of pikeperch aquaculture

Pikeperch is among the candidate species with highest potential for the development of European inland aquaculture. It delivers tasty flesh and can be used for various forms of preparation. Compared to other species, such as common carp (*Cyprinus carpio*), the filet is without intra-muscular spines and easy to process. Since the 1970s, annual capture fisheries of pikeperch have been decreasing to below 21,000 t since 1994 (FAO, 2016). In parallel, market demand and value is attracting necessary investment (8-11 € kg^{-1} farm gate value for

whole fish, up to 50 € kg^{-1} filet at the counter). Consequently, pikeperch farming witnessed a substantial boost during the last decade (Fig. 0.3). Several new farms have been established or are currently under construction and a further increase of production is expected (Mylonas and Robles, 2014; Overton et al., 2015; Steenfeldt et al., 2015).

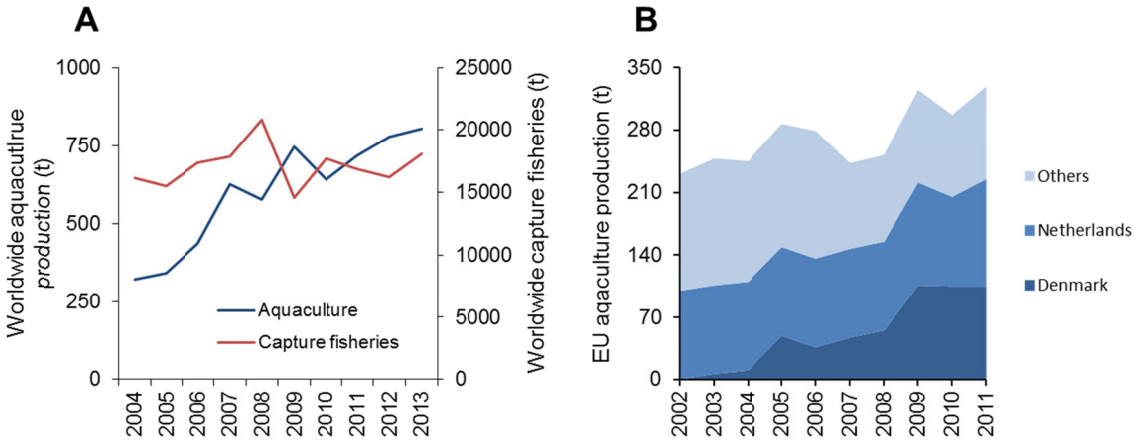


Fig. 0.3. Worldwide aquaculture and capture fisheries production (A) and EU aquaculture production (B) of pikeperch (FAO, 2012, 2016).

Whereas pikeperch are traditionally farmed in ponds, limiting the production to a single annual year-class, a shift towards a technology driven RAS-based production has taken place especially in Central and Northern Europe (Overton et al., 2015; Steenfeldt et al., 2015; Wuertz et al., 2012). This technique has several advantages and is particularly beneficial for pikeperch farming. Optimal growth of pikeperch can be reached at constant temperatures of ~24 °C, which is only feasible in RAS shortening the production of market sized fish from 3 to 5 years in ponds towards 13 to 15 months in RAS (Overton et al., 2015). In parallel, such constant high temperatures in combination with a 24 h photoperiod suppress gonad maturation directing energy to somatic growth (Hermelink et al., 2011, 2013). However, knowledge of species-specific requirements regarding environmental cues (temperature and photoperiod), as well as optimal nutrition and broodstock rearing is a prerequisite for the (year-round) reproduction of fish in RAS. Consequently, combined efforts of research and the commercial sector have been targeting this species during the last two decades. To date, (re)production protocols for pikeperch have been established, which are currently applied by commercial farms and first breeding activities have been launched. Still, the species is far away from domestication and optimization is required to fully overcome its candidate status.

Pikeperch production in RAS is usually based on several separated broodstocks (mixed sexes) with spawners originating from the wild or from on-growing facilities. Each broodstock is adapted to time-shifted photothermal conditions simulating the full annual cycle. Gonad maturation is activated by lowering temperatures to below ~12 °C for a minimum of 3 months in combination with decreasing day length (Hermelink et al., 2011, 2013; Żarski et al., 2015). This can take place at any given time resulting in multiple (out-of-season) spawning events per year. After wintering, final gamete maturation and spawning is induced by elevation of the temperature to ~12 to 16 °C, with or without the use of hormones for stimulation and/or synchronization of spawning (e.g., Müller-Belecke and Zienert, 2008; Steffens et al., 1996; Zakęś and Demska-Zakęś, 2009). Many farms use a 'natural' spawning method by placing artificial nests (e.g., synthetic turf, coconut mats) inside the rearing tanks (Müller-Belecke and Zienert, 2008; Zakęś and Demska-Zakęś, 2009; Żarski et al., 2015). After mating and spawning, fertilized eggs are harvested from the nests. While such 'natural' spawning has benefits, since only a minimum of handling and labor is required, this method often suffers from low fertilization success or the loss of eggs, e.g., eggs being spawned around the nest (Zakęś and Demska-Zakęś, 2009; Żarski et al., 2015). In addition, it does not allow a controlled reproduction of individuals, which is required for breeding programs where proper distribution of genetic material is paramount. As an alternative method, pikeperch spawners are being stripped for *in vitro* fertilization. Despite the obvious advantages, this method is not easy to apply, since timing of stripping is difficult, especially in regard to ovulation to avoid spontaneous release of eggs and/or post-ovulatory ageing, and fish need to be repeatedly handled and anaesthetized often resulting in high mortality of spawners (Zakęś and Demska-Zakęś, 2009; Żarski et al., 2012, 2015).

After de-adhesion treatment of the eggs, e.g., by the use of tannin solution, eggs are transferred to incubation systems (e.g., Zuger-jars) at ~16 °C where hatching takes place on day four post-fertilization (Demska-Zakęś et al., 2005; Żarski et al., 2015). Due to their light and stress sensitivity and the frequent occurrence of irreversible cannibalistic behavior, the rearing of pikeperch larvae and fry is relatively difficult (Steenfeldt, 2015). Again, intensive research has supported the establishment of protocols overcoming major bottlenecks towards large-scale production in RAS (Steenfeldt, 2015). Fingerlings are either transferred or sold to on-growing facilities or are used for restocking measures, since pikeperch is a highly valuable species for recreational angling fisheries.

3 Bottleneck: Gamete quality

For a reliable and constant market supply it is necessary to be able to produce large quantities of fish at any given time of the year. Therefore, the provisioning of high quality gametes in high numbers is a prerequisite for the successful establishment of species in aquaculture (Bobe and Labbé, 2010; Brooks et al., 1997; Kjørsvik et al., 1990; Migaud et al., 2013). In pikeperch, however, variability in gamete quality remains a major bottleneck impeding its propagation (Overton et al., 2015; Schaerlinger and Żarski, 2015).

In other, commonly farmed species variability in gamete quality has been attributed to broodstock rearing and parental traits, such as temperature protocols, parental fish size, the use of hormones, nutrition or handling stress (e.g., for review: Bobe and Labbé, 2010; Brooks et al., 1997; Izquierdo et al., 2001; Migaud et al., 2013; Valdebenito et al., 2013). In parallel, the year-round, out-of-season reproduction has been suggested to exert adverse effects on egg quality (Brooks et al., 1997; Schaerlinger and Żarski, 2015). The manipulation of the sensitive relation of environmental cues and reproduction can result in perturbation of the neuroendocrine pathways, which regulate reproduction (Hermelink et al., 2011, 2013). It was hypothesized that such interference is passed on to the gametes leading to alterations in the developmental potential during early ontogeny (McCormick, 1998). However, there is only limited knowledge on the magnitude and drivers of gamete quality variability in pikeperch.

High quality in eggs is commonly defined as the ability to be fertilized and to subsequently develop into an embryo, which is able to hatch (Bobe and Labbé, 2010). Hence, an oocyte needs to contain all necessary components required for the successful embryonic development. A deficit, as well an excess of designated egg components, e.g., specific fatty acids (FA), nutrients, maternal mRNA, can significantly influence or even prohibit embryogenesis (e.g., Bobe and Labbé, 2010; Bonnet et al., 2007; Schaerlinger and Żarski, 2015). In turn, knowledge of the impact of such components enables the prediction of the developmental potential of an embryo already in an unfertilized egg. In contrast, high quality sperm needs to be able to successfully fertilize an egg, whereas it does not contribute substantially to the egg composition apart from the necessary genetic material. Therefore, sperm quality can be determined through the observation of motility traits, such as velocity or the ratio of moving sperm, enabling the prediction of fertilization success (Alavi and Cosson, 2005, 2006; Bobe and Labbé, 2010; Rurangwa et al., 2004).

These gamete traits are shaped by individual parental characteristics and/or broodstock rearing (e.g., Brooks et al., 1997; Izquierdo et al., 2001; Valdebenito et al., 2013). Perturbation or suboptimal conditions are potentially reflected in the composition of the eggs or influence sperm performance. Such perturbation may be caused by the manipulation of environmental cues or handling induced stress during reproduction. In addition to the inherent gamete characteristics, eggs and sperm are exposed to external influences during artificial *in vitro* fertilization. The required handling of spawners and gametes can potentially exert adverse effects on the quality ultimately alternating developmental success.

The identification of predictive biomarkers of developmental potential is a major research task for the development of aquaculture (Bobe and Labbé 2010; Migaud et al., 2013). Such biomarkers would allow for the comparison of gamete quality across a range of production sites or populations. Also, it is possible to draw conclusions from the observation of gamete attributes regarding the identification of suboptimal spawner characteristics and/or effects of hatchery protocols. Knowledge of these sensitive interactions can help improving and stabilizing production of pikeperch in RAS. However, to date there are only few reports on species-specific biomarkers in pikeperch and observations are almost exclusively limited to sperm traits (Alavi et al., 2015; Schaerlinger and Żarski, 2015).

4 Objectives and aims

The propagation of pikeperch in aquaculture is impeded by high variability in gamete quality associated with low fertilization success and high embryo mortality. There is a lack of information on the effects of broodstock management and maternal traits potentially contributing to this variability. In addition, there is only limited knowledge on species-specific predictive biomarkers in regard to egg morphology and composition, which is of utter importance for successful embryo development. Therefore, the main aims of this thesis were

- to identify potential pitfalls for gamete quality, such as year-round reproduction and currently applied fertilization protocols,
- to further understand the interactions between maternal characteristics and morphological, biochemical, as well as molecular egg parameters and how these properties affect the future developmental potential of the oocytes,
- to evaluate predictive biomarkers of egg quality

in an integrative study under commercial hatchery conditions in pikeperch.

In cooperation with an established RAS-based pikeperch hatchery (AquaPri, Egtved, Denmark), fecundity and developmental rates of eggs and embryos were assessed (Fig. 0.4). Hatching rate was determined as biological endpoint, since free-swimming larvae are exposed to external influences, which lead to an increase in variability exacerbating comparability. Effects of maternal characteristics and broodstock management, as well as biochemical and morphological egg parameters were observed (Fig. 0.5). These parameters were either based on previous reports in regard to gamete quality in other fish species or were identified as potential general biomarkers for cell viability.

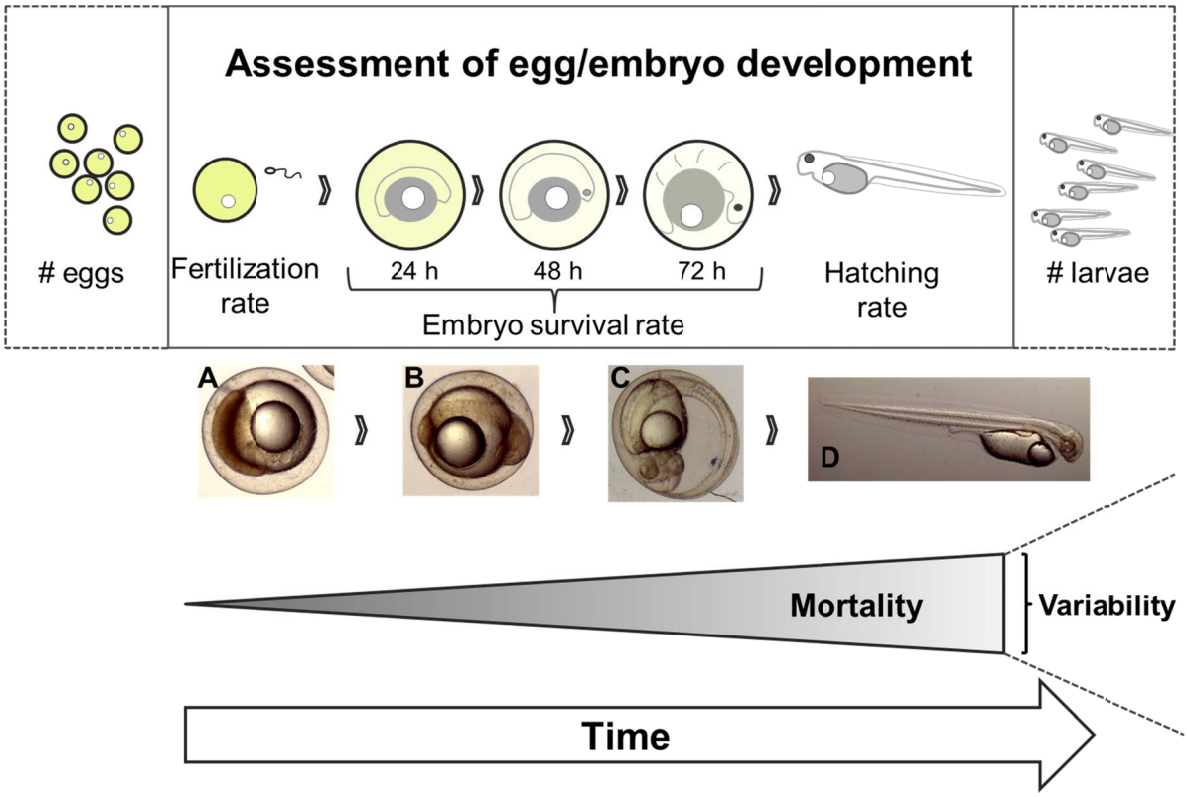


Fig. 0.4. Assessment of the developmental potential of oocytes. Photographic images show an egg few hours post-fertilization (A), a 24 h (B) and 72 h (C) old developing embryo and a freshly hatched larvae (D). (Photographic images by M. Tielmann with his permission)

In contrast to eggs, where timing of ovulation is critical and collection of freshly spawned eggs is difficult, sperm is relatively easy to obtain due to the reproductive biology of pikeperch. Darkened males sit on the nest ready to spawn and may even be repeatedly stripped over the course of a spawning season. However, there is only limited knowledge on

the effects of current hatchery practice regarding the pooling of sperm prior to *in vitro* fertilization or the effects of short-term storage, which is often required until ovulated eggs become available. Therefore, experiments were conducted to evaluate *in vitro* fertilization protocols and to identify and possibly counteract adverse effects of short-term storage. Here, sperm performance was assessed by sperm motility and velocity using computer assisted sperm analysis (CASA).

Generally, these findings deliver insights into the physiological processes involved in variability of gamete quality, how it can be predicted and potentially be optimized. Herewith, they can deliver valuable management advice for pikeperch hatcheries contributing to the propagation of this species in aquaculture.

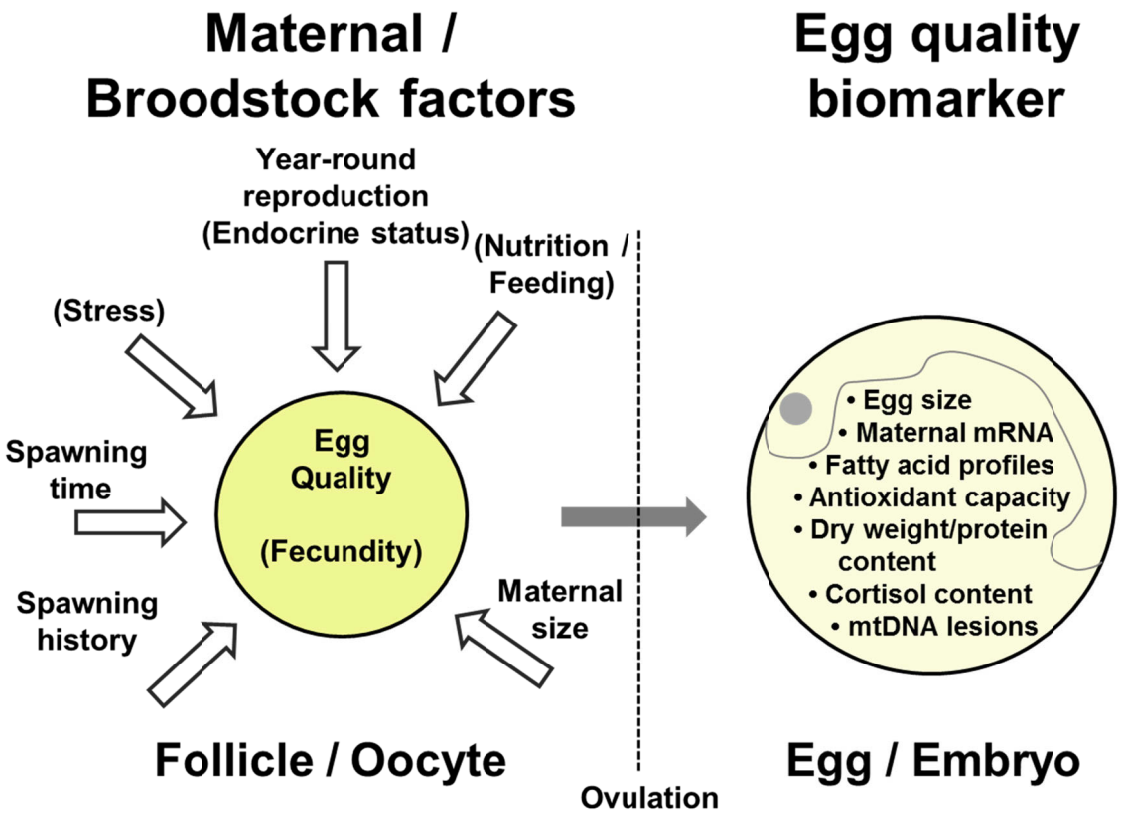


Fig. 0.5. Assessment of effects of maternal and broodstock factors on egg quality, as well as fecundity of pikeperch spawners (left). Bracketed factors were indirectly assessed via the egg composition (cortisol, fatty acid profiles). The assessed biochemical and morphological egg parameters (right) were used to evaluate predictive biomarkers indicating developmental potential of the eggs.

5 Main chapters

This thesis was conducted as part of the research project 'Gamete quality in recirculating aquaculture of pikeperch (*Sander lucioperca*)' (original german title: „Einfluss der jahreszeitlich unabhängigen Reproduktion auf die Qualität von Gameten und frühen Lebensstadien des Zanders (*Sander lucioperca*)") funded by the German Research Foundation (grant number: DFG KL 745/6-1). It is a cumulative work based on three research papers (chapters I, II and IV). Each of these three chapters includes an introduction, material and methods, results, discussion and conclusion, as well as references section. Paper II and IV are published/accepted manuscripts reprinted with the permission of the publisher. For a consistent layout of this thesis, parts of the text were reformatted, tables and figures were renumbered. In chapter III, a synthesis of the first two papers dealing with egg quality is provided, which includes additional results and their discussion regarding interactions of oxidative stress and other parameters of egg composition, as well as maternal characteristics. Following these four chapters, a general discussion is provided, knowledge gaps are discussed and possible applications for hatchery management are highlighted.

Chapter I: Schaefer FJ, Overton JL, Krüger A, Kloas W, Wuertz S (submitted manuscript) Broodstock management and egg quality in pikeperch (*Sander lucioperca*).

Chapter II: Schaefer FJ, Overton JL, Wuertz S (2016) Pikeperch *Sander lucioperca* egg quality cannot be predicted by total antioxidant capacity and mtDNA fragmentation. Animal Reproduction Science 167:117-124.
DOI: 10.1016/j.anireprosci.2016.02.016

Chapter III: Synthesis: Interactions of oxidative stress, maternal characteristics and biochemical egg composition in pikeperch

Chapter IV: Schaefer FJ, Overton JL, Bossuyt J, Żarski D, Kloas W, Wuertz S (accepted manuscript) Management of pikeperch *Sander lucioperca* sperm quality after stripping. Journal of Applied Ichthyology (in press)

Chapter I

Broodstock management and egg quality in pikeperch (*Sander lucioperca*)

Broodstock management and egg quality in pikeperch (*Sander lucioperca*)

Fabian J. Schaefer, Julia L. Overton, Angela Krüger, Werner Kloas and Sven Wuertz

Manuscript submitted to the journal Biology of Reproduction

Abstract

Reproduction of temperate fish species is regulated by external and endogenous signals. It was hypothesized that the manipulation of environmental cues, as often practiced in aquaculture, may interfere with endogenous signals affecting the reproductive success. In an integrative study, (I) an array of commonly studied parameters associated with egg quality, (II) the effect of year-round reproduction and broodstock characteristics on egg composition and development and (III) predictive biomarkers were evaluated in 41 egg batches of commercially reared pikeperch (*Sander lucioperca*). Fertilization, embryo survival at 24, 48, 72 h and hatching rate were used to assess developmental potential. Substantial variability in developmental rates could be explained (47.1% fertilization; 58.2% 24 h, 47.0% 48 h, 43.9% 72 h survival; 46.6% hatching; 88.9% hatched larvae) by fecundity, maternal length, egg size and specific fatty acids (polar 20:5(n-3), 15:0, 18:0). The inherent oocyte composition exerted the highest influence during early development until 48 h. Fatty acid profiles were identified as major integrative parameters affecting oocyte development and being associated with other egg features, especially egg cortisol content, as well as fecundity and maternal traits (spawning history and time, female length). Other parameters had no direct effect (cortisol, prohibitin2 mRNA) or only a weak impact (egg dry weight content). Conclusively, female length, rather than endogenous zeitgeber, spawning history (first-time versus successively stripped) and time of spawning negatively influenced egg quality. An optimal maternal length of ~65 – 70 cm was associated with high fecundity and egg quality. In turn, specific egg characteristics can be used to predict the developmental potential of the oocytes.

1 Introduction

Aquaculture is among the fastest growing sectors of food production worldwide. However, production in the European Union (EU) does not participate in this trend and production is stagnating, with few exceptions. In contrast, the EU is a major fish market depending on imports [1]. The traditional aquaculture techniques of freshwater fish, mainly carp and trout species, in the EU are predominantly pond based resulting in spatial and environmental limitations regarding an up-scaling of production. Consequently, the latest reform of the EU common fisheries policy aims at an increase of regional production applying a technology driven approach through the implementation of recirculating aquaculture systems (RAS) [2]. Since site-independent intensive farming in RAS is challenged by the economic feasibility, production efficiency needs to be improved. In RAS, production times can be shortened, e.g., by constant high temperatures, which in parallel suppress gonad maturation, directing available energy to somatic growth [3]. Through the full control of environmental conditions reproduction can be triggered at any given time of the year [4, 5].

Pikeperch (*Sander lucioperca*) is among the species with highest potential for RAS-based aquaculture production. Under natural conditions, pikeperch reproduce only once per year in the wild during spring limiting the availability of stocking material [6]. Reproduction can be achieved by photothermal control of sexual maturation [3, 7], with or without the use of hormone treatments [8, 9]. Thus, by rearing and managing several broodstocks separately, spawning can be achieved throughout the year. Often, spawners are used successively over consecutive years. However, fertilization failure and high variability in embryo survival are impeding the culture of pikeperch and other emerging species [10, 11]. Therefore, reaching and maintaining a constant, reliable supply of high quality eggs in large quantities are important aims for reproductive management in aquaculture [12-14].

Egg quality is commonly defined as the ability of an oocyte to be fertilized and to develop into a normal embryo, which is able to hatch successfully. This ability is dependent on the morphological traits, as well as biochemical and molecular composition of the oocyte, which in turn is largely determined maternally. In contrast, paternal influences are limited to fertilization and nuclear-genetic information. Variability in egg composition and hence, in quality has been mainly attributed to broodstock rearing and maternal traits. Several factors acting on the eggs on the parental level are known including temperature, size of female

spawners, hormone treatment, broodstock nutrition and handling stress [12-16]. However, the manipulation of the endogenous zeitgeber of reproduction as caused by manipulation of environmental cues during year-round reproduction is supposed to exert additional adverse effects on reproductive performance [11, 13]. Still, there is a substantial lack of knowledge in regard to the effects of year-round, out-of-season spawning on egg quality.

Similar to other species, the neuroendocrine control of reproduction in pikeperch is closely linked to exogenous environmental factors [3]. The manipulation of these interactions can result in perturbation of this sensitive relation [17, 18]. Maternal effects are passed on to the offspring alternating the developmental potential of embryos [19]. For example, there is increasing evidence that stress [20-22], as well as steroidogenesis [23, 24] are linked to certain aspects of nutrition, such as designated highly unsaturated FA (HUFA). In turn, the HUFA composition of broodstock diets is reflected in the eggs affecting the future development and egg FA profiles are suitable biomarkers of egg quality as shown in several species, including freshwater percids [25-27, 28]. Generally, FA are acknowledged as integrative parameters being associated with bioenergetic aspects, as well as various physiological and endocrine mechanisms. For example, HUFA are involved in steroidogenesis [15, 29, 30], final oocyte maturation [31] and timing of spawning [32], are integrative components of cell membranes and precursors of eicosanoids [33-35]. Similarly, it was shown that stress and cortisol as primary stress response factor are tightly coupled to maturation physiology in fish affecting timing of spawning, reproductive fitness and subsequently egg quality [36]. Consequently, processes on the maternal level modulate embryo development via alternations of the biochemical composition of the eggs. Vice versa, observing these traits in eggs allows for drawing conclusions on the state of the maternal organism. However, it remains unknown to what extent these integrative parameters might change in regard to endogenous zeitgeber during out-of-season reproduction.

In addition, the identification and evaluation of egg quality biomarkers, which are indicative for the future developmental potential is commonly recognized as important research task in fish physiology [12, 14]. Benefits arise from better insights into mechanisms determining egg quality and thus may help to improve hatchery technology, as well as broodstock management. Secondly, reliable biomarkers would allow for a comparative approach across a range of production sites or populations, further increasing the knowledge and improving the currently applied practice of pikeperch farming. To date, indicators of

pikeperch egg quality are predominantly based on oocyte morphology [11] or failed to predict embryo development studying markers of oxidative stress [37]. In other freshwater percids, such as Eurasian perch (*Perca fluviatilis*) or walleye (*Sander vitreus*), FA (e.g., ratios of n-3 and n-6 HUFA) were used to predict egg quality [25, 27, 28]. However, these parameters are still controversially discussed and there is an urgent need for species-specific biomarkers.

We conducted a study in co-operation with an established RAS-based pikeperch producer (AquaPri, Denmark) and examined egg batches of 41 pikeperch covering six independent spawning seasons over the course of three years using four distinct, separated broodstocks. Fertilization rate, embryo survival at 24, 48 and 72 h, as well as hatching rate on day four post-fertilization were assessed to characterize the future developmental potential of the eggs. The effects of female size, spawning history (fish that have been stripped before or fish being stripped for the first time) and the time of spawning (days) into each season were analyzed to further understand the influence of maternal broodstock characteristics, as well as the underlying causes of variability in egg quality. In addition to egg diameter, integrative parameters considered were egg cortisol concentrations, profiles of polar and neutral FA. To date, the optimal FA composition of eggs in pikeperch supporting fertilization and optimal embryonic development is unknown. Furthermore, the relative abundance of prohibitin2 (*Phb2*) mRNA was assessed, which was associated with embryo mortality in rainbow trout (*Oncorhynchus mykiss*) [38]. Protein and dry weight (DW) content were determined as markers of energy content and osmoregulation [39, 40].

We used this dataset (I) to evaluate an array of egg parameters associated with egg quality in an integrative study for the first time in pikeperch, (II) to determine the effect of year-round reproduction and broodstock characteristics on pikeperch egg composition and quality under commercial hatchery conditions and (III) to identify predictive biomarkers. These results allow drawing conclusions on physiological coherences in the oocyte. Furthermore, they can be used to identify potential points of leverage to optimize broodstock management to increase egg quality in the future.

2 Material and Methods

2.1 Sampling

All samples originate from a commercial pikeperch farm (AquaPri, Egtved, Denmark) in accordance with EU and National legislation for animal welfare in fish production. The breeders (mixed sexes) were divided into four separated broodstock groups. Fish were fed the same diet at apparent satiation and were always handled by the same experienced personal to standardize procedures and minimize handling stress. Maturation was induced once per year in each broodstock by four months of wintering (<14 °C) and subsequent warming to ~16 °C to support maturation and to induce ovulation (no hormone treatment). The temperature protocol was identical for all four groups, but applied in intervals of three months providing four distinct spawning seasons per year (spring, summer, autumn and winter). Over the course of three consecutive years (2013-2015), batches of 41 females were sampled covering six spawning seasons (Summer 2013, n = 6; Autumn 2013, n = 8; Winter 2014, n = 6; Summer 2014, n = 7; Winter 2015, n = 8; Spring 2015, n = 6). The spawning history of most of these 41 females was known, providing 16 spawners stripped for the first-time and 20 spawners with spawning experience (fish that have been stripped successively).

At time of ovulation, fish were anesthetized (Kalmagin 20%; Centrovet), total body length was measured to the nearest cm and eggs were stripped. The date of first stripping was referred to as beginning of the respective spawning season for the determination of the time of spawning for each female into the season. Total volume of eggs was determined to the nearest ml and total fecundity was calculated using egg counts of a 2 ml subsample. Samples of unfertilized eggs were taken, frozen, transported to the lab in liquid nitrogen and stored at -80 °C.

The remaining eggs were fertilized with freshly stripped sperm after a visual sperm quality check (sperm activation visible), transferred to Zuger-jars and incubated at ~15 to 16 °C. A minimum of 50 eggs were monitored in triplicates at 2, 24, 48, 72 h after fertilization and prior to hatching (day 4) for the determination of fertilization rates, respective embryo survival and hatching rates (%) using a Stereozoom IT-TR microscope (Gundlach). The total number of hatched larvae per female was calculated (number of hatched larvae = total fecundity x hatching rate/100). Due to hatchery related operations batches with extremely high mortality were removed before determining hatching.

2.2 Fatty acid (FA) analysis

A subset of ~100 mg eggs (wet weight, WW), was freeze-dried in an Alpha 1-4 LOC-1M (Christ) for 48 h and the ratio of dry weight (DW) to WW (%) was recorded. For FA analysis, ~3 mg (DW) eggs were extracted and measured according to Boëchat et al. [41] with the following modifications. Samples were homogenized for 2 min with ultra-sonication and 20 mg butylhydroxytoluol (BHT; Carl Roth) were added to 100 ml of 2:1 chloroform-methanol (v:v). An aliquot of 1.2 ml of the upper layer was evaporated at 40 °C with a rotary evaporator (Rotavapor R200 and heating bath B490; Buchi) and re-suspended in 500 µl 2:1 chloroform-methanol (v:v). Prior to methylation, solid phase extraction of polar and neutral FA was performed using Strata NH2 (55 µm; 70 Å) 1000 mg/6 ml columns (Phenomenex), which were pre-conditioned with 5 ml of 2:1 chloroform-methanol (v:v). First, neutral FA were extracted with 20 ml of acetone (Carl Roth). After drying of the column, polar FA were extracted with 20 ml methanol (Carl Roth). Extracts were evaporated as described above.

After methylation of each fraction, 4.5 ml hexane (Carl Roth) were added and incubated for 15 min under continuous shaking. The solution was centrifuged for 5 min at 2300 g and the upper layer containing the lipid extract was transferred to a glass tube. This extraction was repeated twice with 2 ml hexane. Subsequently, 15 ml of potassium bicarbonate solution (2.8 g L^{-1} $KHCO_3$; Sigma Aldrich) were added to the extracts, briefly mixed and the hexane phase was transferred to a 100 ml pear-shaped flask. After evaporation, the FA were re-suspended in 200 µl hexane and stored at 4 °C until measurement in an Agilent 6890N gas chromatograph (Agilent) equipped with an Agilent 5973N mass selective detector (Agilent) and a fused silica capillary column (J&W CP-Sil 88 for FAME; Agilent). Detection and determination thresholds were 0.1 and 0.4 µg mg^{-1}, respectively. For statistical analysis, values below the quantification limit were set to 0.05 µg mg^{-1}. The divergence between determination threshold and the set value of 0.05 µg mg^{-1} accounted on average for 3.4% difference in total FA content.

2.3 Cortisol

For determination of cortisol concentrations, ~60 mg of eggs were manually crushed with a pistil for 2 min. Extraction and analysis of 30 µl of crushed eggs were performed according to Hermelink et al. [17] using cortisol-specific enzyme-linked immunosorbent assays (ELISA;

IBL) according to the manufacturers instructions. Each sample was measured in duplicate and concentration was calculated from the dilution series. Recoveries determined upon spiking were above 91%.

2.4 Protein content

For the protein assays, ~30 mg of eggs were manually crushed with a pistil for 2 min in 500 µl of distilled water, assessing a subsample of 20 batches. Aliquots of 5 µl were diluted 1:150 in distilled water. Protein content (% of egg wet weight) was determined in duplicate using Roti-Quant (Roth) detergent according to the manufacturers instructions in an Infinite M200 Pro microplate reader (Tecan) at 590 nm and calculated from a dilution series of bovine serum albumin (BSA; Sigma-Aldrich).

2.5 RT-QPCR

For mRNA extraction, samples of unfertilized eggs were incubated in RNAlater reagent (Qiagen) for 24 h at 4°C and subsequently stored at -20 °C. The extraction was performed according to Kroupova et al. [31] with a TissueLyser (Qiagen). The RNA concentrations were measured in triplicate by UV absorption spectrometry (Nanodrop ND-1000 spectrophotometer; Thermo Fisher Scientific) and RNA was diluted to a final concentration of 20 ng μl^{-1}. For a subset of seven samples, RNA integrity number (RIN) was determined with a RNA 6000 NanoLabChip in an Agilent 2100 Bioanalyzer (Agilent Technologies). The RIN ranged from 9.45 to 9.90 confirming RNA integrity. Reverse transcription was performed using the Affinity Script Multi Temperature reverse transcriptase (Agilent).

For the identification of *Phb2*, conserved primers were designed using sequence information of teleost species and specific amplification was confirmed by direct sequencing and analysis using the generated alignment. Primers for RT-QPCR were designed and subsequently validated by direct sequencing. Primers for ribosomal protein L8 (*Rpl8*) were derived from Hermelink et al. [17]. All RT-QPCR reactions were determined in duplicate in a Mx3005 cycler (Agilent Technologies) and product specificity was confirmed by melting-curve analysis. Temperature protocols differed for *Phb2* [3 min initial degeneration at 96 °C, followed by 40 cycles of degeneration at 96 °C, annealing at 65 °C and elongation at 72 °C with 20 s per step] and *Rpl8* [3 min initial degeneration at 96 °C, followed by 40 cycles of 15

s degeneration at 96 °C and 20 s annealing at 62 °C]. Amplification was carried out using 10 µl Brilliant III SYBR Master Mix (Agilent), 12.5 ng of DNA, 0.4 µM of each primer and 6 µl of PCR grade water (Qiagen) in 20 µl total reaction volume. The RT-QPCR specifications are summarized in table 1.1. Relative expression of *Phb2* was calculated as $2^{(\mathrm{Ct}\ Phb2 - \mathrm{Ct}\ Rpl8)}$.

Table 1.1. Specifications for the RT-QPCR assay for prohibitin2 (*Phb2*), using ribosomal protein L8 (*Rpl8*) as a housekeeping gene, forward (f) and reverse (r) primers, primer-specific product length in base pairs (bp), GenBank accession number (GenBank #), annealing temperature (TA) and PCR efficiency (Eff).

Target gene	Primer	5'-3' sequence	TA [°C]	Length [bp]	GenBank #	Eff [%]
Phb2	f	AATGTTGATGCAGGTCACCG	65	289		99.4
	r	TCTGTGGTGATGGATGGCAG				
Rpl8	f	GTTATCGCCTCTGCCAC	62	167	HQ259050	96.5
	r	ACCGAAGGGATGCTCAAC				

2.6 Egg size

Egg diameter of 10 eggs was determined with ImageJ software (version 1.44; National Institute of Health, USA) and an Olympus SZH binocular attached to a XC50 CCD camera. Each egg was assessed in triplicate.

2.7 Data analysis

If not otherwise mentioned, data are presented as mean ± standard deviation (SD). Data were checked for normality with Kolmogorov-Smirnov normality test. For group comparison, one-way ANOVA with Tukeys post-hoc test (parametric data) or Kruskal-Wallis test with Dunns post-hoc test (nonparametric data) were used. For the comparison of two groups, parametric Students t-test or nonparametric Mann-Whitney test were carried out. Correlation analysis was performed with Pearsons (r) and Spearmans (ρ) correlation. Pearsons correlation is only presented if there was no significant result using Spearmans correlation. Linear

regression and multiple-linear regression was performed for the evaluation of biomarkers. For regression analysis, percentage data (fertilization, embryo survival and hatching rates) were arcsine transformed. Normal distribution of regression residuals was checked with Shapiro-Wilk normality test. Data analysis was performed with PRISM software (version 4.03; GraphPad) or SPSS (version 22; IBM).

3 Results

3.1 Fecundity and developmental performance: Fertilization, embryo survival and hatching

The mean ± SD volume of eggs per female spawner was 408 ± 143 ml (n = 40), ranging from 140 to 740 ml. Total fecundity was 495 ± 175 $*10^3$ eggs (n = 39), ranging between 120 and 840 $*10^3$ eggs respectively. On average, each ml contained 1,195 ± 180 eggs. Fertilization ranged from 40.0 to 100% with an average of 89.2 ± 16.8% (Fig. 1.1). Embryo survival dropped from 85.7 ± 15.9% at 24 h to 79.4 ± 19.8% at 48 h and was 78.8 ± 16.3 at 72 h. Hatching rate observed on day 4 was 77.9 ± 15.5% on average ranging from 40.0 to 99.0%. Thus, highest mortality occurred during the first 48 h. The mean number of hatched larvae per batch was 380,363 ± 164,458 (n = 29). Total fecundity and the number of hatched larvae were strongly correlated ($\rho = 0.86$; $p < 0.001$). Hatching rates were positively related to fecundity ($\rho = 0.33$; $p < 0.05$) and not associated with fertilization rate or embryo survival after 24, 48 and 72 h.

Three batches underwent post-ovulatory ageing before stripping (4 to 12 h). Only one batch could be successfully fertilized (40.0% fertilization rate), but no embryos survived after 24 h. There were no signs for differences in egg composition or diameter between these aged eggs and the other 38 egg batches, which were stripped at time of ovulation. Obtained data of developmental rates and egg quality parameters of aged eggs were excluded from correlation and multiple regression analysis.

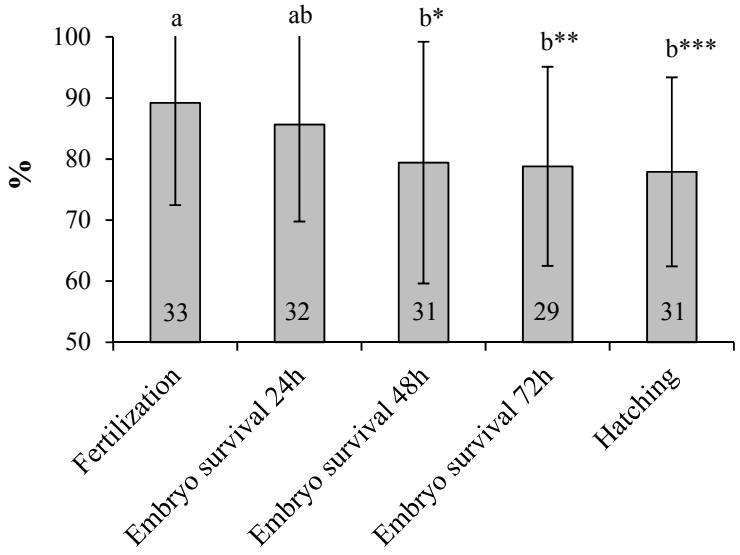


Fig. 1.1. Fertilization, embryo survival and hatching rates for female pikeperch spawners. Survival is not accumulated but presented for each time point. The number of batches considered is given per column. Whiskers indicate the standard deviation. Significant differences in-between groups are indicated by lower case letters. The level of significance is indicated by asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

3.2 Egg parameters

Results of all egg batches were combined delivering overall means of biochemical egg composition and egg size. The results of the FA analysis and correlation with fecundity and egg development are summarized in table 1.2. The total FA content (polar and neutral) was 116.5 ± 25.7 µg mg^{-1} DW with a minimum of 66.1 and maximum of 171.7 µg mg^{-1}. Polar FA ranged from 9.3 to 30.1%. Especially unsaturated polar FA were strongly correlated with fecundity and the number of hatched larvae.

The results of the egg analyses and correlations with fecundity and egg development are summarized in table 1.3. Correlation analyses within parameters and broodstock, as well as spawning characteristics are summarized in table 1.4. The mean egg diameter was 1.19 ± 0.10 mm ranging from 0.99 to 1.45 mm. There was a positive correlation between egg size and fecundity. A larger egg size had a negative impact on embryo survival (24, 72 h), as well as hatching rate. Individual FA contents were independent of the egg diameter, but there was a positive correlation of egg size and ratio of neutral docosahexaenoic acid (DHA) to eicosapentaenoic acid (EPA).

Table 1.2. Mean absolute (µg mg^{-1} dry weight) ± SD and relative (%) abundance of major fatty acids (FA)* representing 98.6 % of total FA found and selected ratios in polar and neutral fatty acids for all 41 batches of pikeperch eggs. Significant correlations of FA with fecundity and developmental rates are listed including the correlation coefficient (Spearman or Pearson, the latter in brackets). The level of significance is indicated by asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Fatty acid	Mean	± SD	% of total FA	Fecundity (*1000 eggs)	Fertilization rate (%)	Embryo survival (%)		# hatched larvae
						24h	72h	
Polar								
14:0	1.19	± 0.33	1.0					
15:0	0.40	± 0.28	0.3			(0.34*)		
16:0	9.43	± 2.20	8.1					
16:1(n-7)	0.27	± 0.25	0.2	0.39**				0.39*
18:0	4.21	± 1.40	3.6		-0.31*	-0.37*	(-0.32*)	
18:1(n-9)	0.68	± 0.43	0.6	0.57***				0.50**
20:5(n-3), EPA	0.80	± 0.38	0.7	0.54***	(0.30*)	(0.32*)		0.49**
22:6(n-3), DHA	2.76	± 1.34	2.4	0.46**				0.34*
Sum SFA	15.59	± 3.89	13.4					
Sum MUFA	1.40	± 1.07	1.2	0.40**				0.40*
Sum n-3 HUFA	3.56	± 1.69	3.1	0.51***				0.41*
DHA/EPA	3.32	± 0.47	3.5					
Total polar FA	20.68	± 5.24	17.8					
Neutral								
14:0	1.97	± 0.45	1.7					
16:0	13.77	± 3.96	11.8					
16:1(n-7)	9.95	± 3.63	8.5					
18:0	9.57	± 4.02	8.2					
18:1(n-9)	13.34	± 4.26	11.5	(0.30*)				
18:2(n-6)	9.16	± 3.65	7.9					
18:3(n-3)	1.36	± 0.56	1.2	(0.31*)				
20:4(n-6), ARA	0.96	± 0.38	0.8					
20:5(n-3), EPA	6.60	± 2.48	5.7					
22:6(n-3), DHA	26.30	± 9.25	22.6					
Sum SFA	26.43	± 9.01	22.7					
Sum MUFA	24.94	± 8.40	21.4					
Sum n-3 HUFA	34.27	± 11.62	29.4					
n-3/n-6	3.54	± 1.02	3.4					
DHA/EPA	4.17	± 1.14	4.0		(-0.36*)	(-0.40*)		
EPA/ARA	6.89	± 1.20	6.9					
Total neutral FA	95.76	± 25.39	82.2					

Note: SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; HUFA, highly unsaturated fatty acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; ARA, arachidonic acid.

* Other fatty acids routinely found, but generally below quantification limit of 0.4 µg mg^{-1} dry weight: 12:0, 17:0, 20:0, 20:1(n-9), 20:2 (unknown isomer), 22:0, 22:1(n-9), 24:0, 24:1 (unknown isomer)

Table 1.3. Mean ± SD of biochemical and molecular egg parameters, as well as fish size and the day of the spawning season for 41 batches of pikeperch eggs*. Significant correlations with fecundity and developmental rates are listed including the correlation coefficient (Spearman or Pearson, the latter in brackets). The level of significance is indicated by asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

	Mean	± SD	Fecundity (*1000 eggs)	Fertilization rate (%)	Embryo survival (%)			Hatching rate (%)
					24h	48h	72h	
Fish size (cm)	69.4	± 5.7	0.31*	-0.44**	-0.54***	-0.37*	-0.33*	
Days into season (d)	11.9	± 9.4		-0.34*	-0.33*			
Dry weight content (%)	21.0	± 4.0			-0.37*			
Average egg size (mm)	1.19	± 0.1	(0.30*)		-0.41*		-0.42*	-0.41*
Phb2 (relative expression to *Rpl8*)	2.21	± 1.3						
Protein content* (%)	29.8	± 4.6						
Cortisol (ng ml^{-1})	85.4	± 49.5	(-0.33*)					

*Protein content of egg wet weight was determined in a subset of n = 20 egg batches.

Egg cortisol content was highly variable ranging from 22.7 to 293.2 ng ml^{-1} and was negatively correlated with the total fecundity. Similar to the relative expression of *Phb2* (min: 0.1; max: 6.8), embryo development was not influenced by egg cortisol concentrations. There were several significant negative correlations between cortisol and FA, as well as positive correlations between *Phb2* and neutral FA. As in egg size, there was a positive correlation of cortisol with the ratio of neutral DHA/EPA.

A strong correlation between egg DW and protein content was recorded in the batches ($r = 0.73$; $p < 0.001$; $n = 20$). Therefore, DW content was chosen as proxy for protein content for further analysis. The DW content was negatively correlated with embryo survival at 24 h, as well as polar FA 14:0 and 15:0, but positively correlated with polar FA 16:1(n-7) and 18:1(n-9).

Table 1.4. Correlations of observed egg parameters and broodstock characteristics. Significant correlations are listed including the correlation coefficients (Spearman or Pearson, the latter in brackets). The level of significance is indicated by asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

	Parameter	Fish size (cm)	Days into season (d)	% dry weight content	Average egg size (mm)	*Phb2* (relative expression to *Rpl8*)	Cortisol (ng ml^{-1})
	Phb2 (relative expression to *Rpl8*)		(-0.28*)				
	Cortisol (ng mg^{-1} wet weight)	(-0.34*)					
Polar FA	14:0			-0.33*			
	15:0		-0.34*	-0.36*			
	16:1(n-7)	(0.31*)		(0.30*)			-0.55***
	18:1(n-9)	0.42**		(0.29*)			-0.37*
	20:5(n-3), EPA						-0.36*
	22:6(n-3), DHA	0.29*					-0.39**
	Sum MUFA	0.29*					-0.44**
	Sum n-3 HUFA						-0.38**
	Total polar FA						-0.27*
Neutral FA	16:1(n-7)					0.34*	
	18:1(n-9)					0.34*	-0.30*
	18:2(n-6)					0.43**	
	18:3(n-3)		(-0.30*)			0.35*	
	20:4(n-6), ARA		-0.28*			0.36*	-0.35*
	20:5(n-3), EPA					(0.40**)	-0.38*
	22:6(n-3), DHA					0.36*	
	Sum MUFA					0.38*	
	Sum n-3 HUFA					0.35*	
	n-3/n-6				0.35*		0.33*
	DHA/EPA				(0.32*)		
	Total neutral FA					0.34*	

Note: FA, fatty acids; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; HUFA, highly unsaturated fatty acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; ARA, arachidonic acid.

3.3 Maternal influences

There were minor significant differences in fecundity, fertilization, embryo survival and hatching between the seasonal and the out-of-season reproductions studied. Once a significant difference in fertilization and once in survival after 24 h (Dunns multiple comparison: $p < 0.05$) was detected between respective seasons (Fig. 1.2). The last sampled season in spring 2015, which represented the natural spawning season of pikeperch, showed the overall highest variability in developmental rates. Two spawning seasons, autumn 2013 and winter 2015, showed very high fertilization, embryo survival and hatching. Here, hatching rates were > 12% higher compared to all other seasons.

There was no difference in average fish size between spawning seasons, but the total fecundity differed (ANOVA: $F = 5.53$; $p < 0.001$). Especially the three spawning seasons from autumn 2013 to summer 2014, which were comprised of intermediate sized fish (~70 cm) showed significantly higher number of eggs compared to other seasons. The number of hatched larvae was less variable (ANOVA: $F = 3.05$; $p < 0.05$) with only autumn 2013 producing on average a significant higher number of larvae (515,618) compared to summer 2013 (291,600). When looking at egg parameters in-between the six observed spawning seasons, the only significant differences occurred in FA profiles. Still, there were only minor differences in single FA occurrence (polar FA 15:0, 18:0, EPA, total polar n-3 FA) between individual spawning seasons. The only major difference in egg composition, which affected more than one pair of spawning seasons could be detected in the ratio of neutral DHA/EPA (ANOVA: $F = 6.07$; $p < 0.001$). The ratio was significantly elevated in spring 2015 compared to all but one other seasons (Fig. 1.3). In polar DHA/EPA, the spring 2015 season also showed the highest ratios, but the difference was not significant (ANOVA: $F = 2.47$; $p = 0.055$).

Mean fish size was 69.4 ± 5.7 cm ranging from 56 to 80 cm. An increase of fecundity with fish size up to 75 cm could be observed. For larger females, fecundity decreased (Fig. 1.4). Best model fit was a quadratic function (Total fecundity*1000 = 149*fish size – fish size^2 - 4895; $R^2 = 0.14$; $p = 0.073$). Maternal length was negatively correlated with fertilization and embryo survival, but not with hatching rate or the number of hatched larvae. Here, the lowest observed rates of fertilization and embryo survival (up to 24 h) were observed in fish > 74 cm. The egg size was independent of fish length ($\rho = 0.18$; $p = 0.13$),

but there were significant correlations between fish size and cortisol levels and several polar FA (cf. table 1.4).

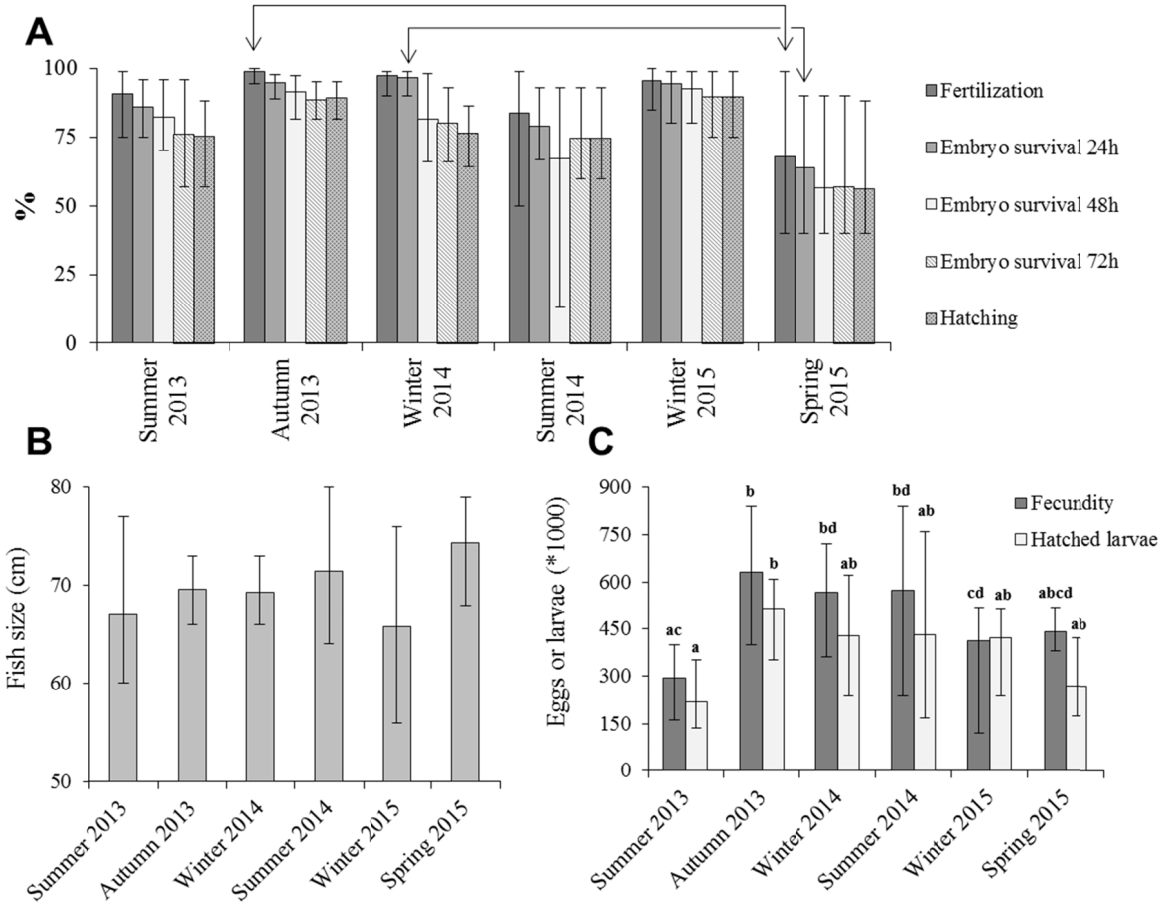


Fig. 1.2. Development (A), fish size (B) and total fecundity and number of hatched larvae (C) for all observed female pikeperch spawners grouped by spawning seasons (summer 2013, n = 6; autumn 2013, n = 8; winter 2014, n = 6; summer 2014, n = 7; winter 2015, n = 8; spring 2015, n = 6). Survival is not accumulated but presented for each time point. Significant differences (Dunns (A) or Tukeys (C) multiple comparison: $p < 0.05$) in-between seasons are indicated by arrows (A) or lower case letters (C). Whiskers indicate the minimum and maximum values.

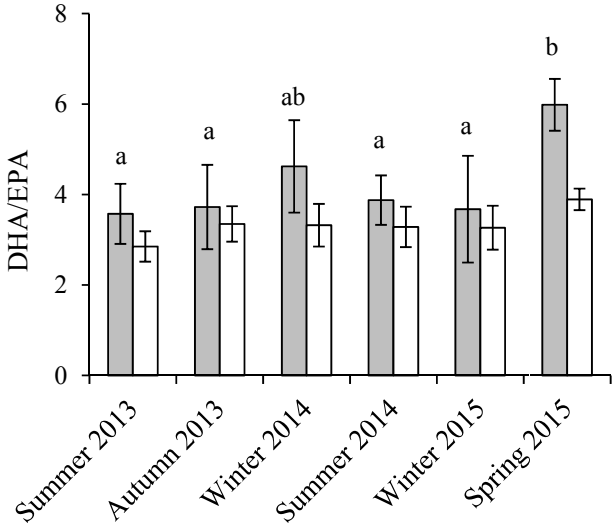


Fig. 1.3. Average ratios of neutral (grey) and polar (white) docosahexaenoic (DHA) to eicosapentaenoic acid (EPA) grouped by spawning seasons. Whiskers indicate the standard deviation. Significant differences in-between groups are indicated by lower case letters (Tukeys multiple comparison: $p < 0.01$).

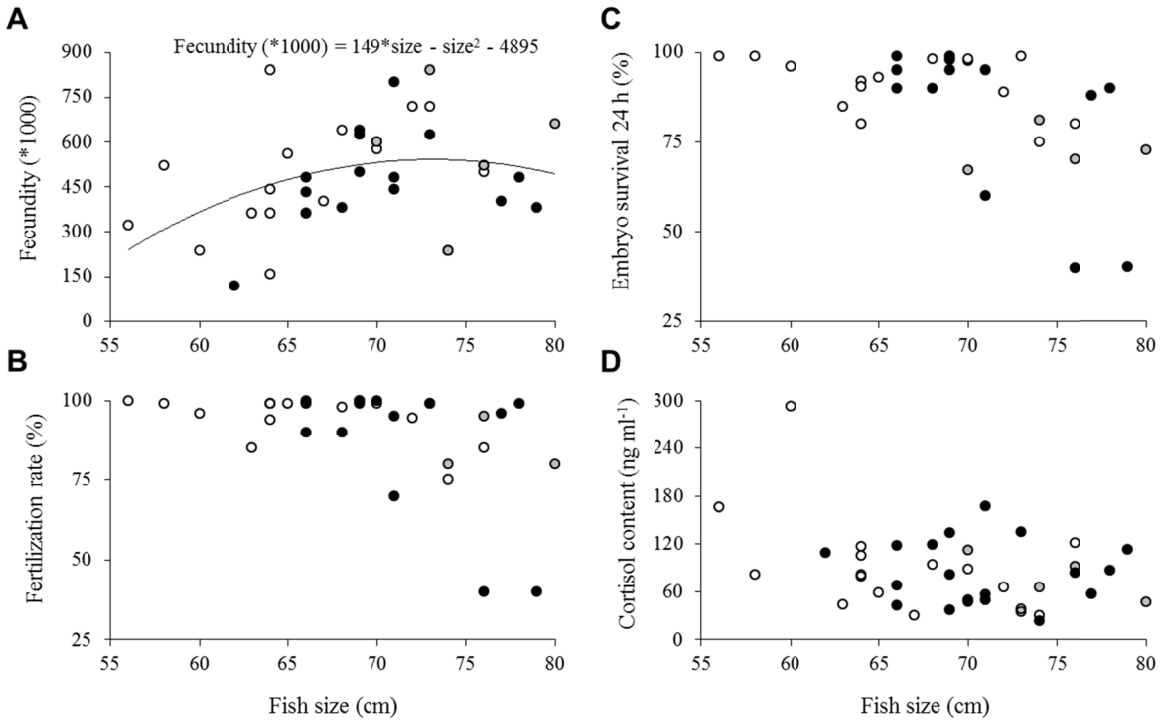


Fig. 1.4. Correlation of total fecundity (A), fertilization rate (B), embryo survival at 24 h (C) and cortisol content (D) versus fish size of spawners stripped for first-time (open circles) and those with spawning experience (black circles), as well as for fish with unknown spawning experience (grey circles). Survival is not accumulated but presented for each time point.

Successively stripped spawners were larger compared to first-time spawners (Students t-test: $F = 1.63$; $p < 0.01$) with an average size ± SD of 70.8 ± 4.5 and 66.1 ± 5.8 cm, respectively, but there was no difference in fecundity or the number of hatched larvae. Upon stripping, mean performance of the respective spawners was not substantially affected, but individual variation of developmental success increased (Fig. 1.5). The only significant differences between first-time and successively stripped spawners could be detected in the FA composition. Successively stripped spawners had a significantly elevated ratio of polar (Students t-test: $F = 1.24$; $p = 0.01$) and neutral (Students t-test: $F = 2.84$; $p < 0.01$) DHA/EPA, whereas spawners stripped for the first-time had higher neutral FA 18:2(n-6) (Students t-test: $F = 1.80$; $p < 0.01$), arachidonic acid (ARA; Students t-test: $F = 1.22$; $p < 0.01$) and EPA (Students t-test: $F = 1.38$; $p < 0.001$).

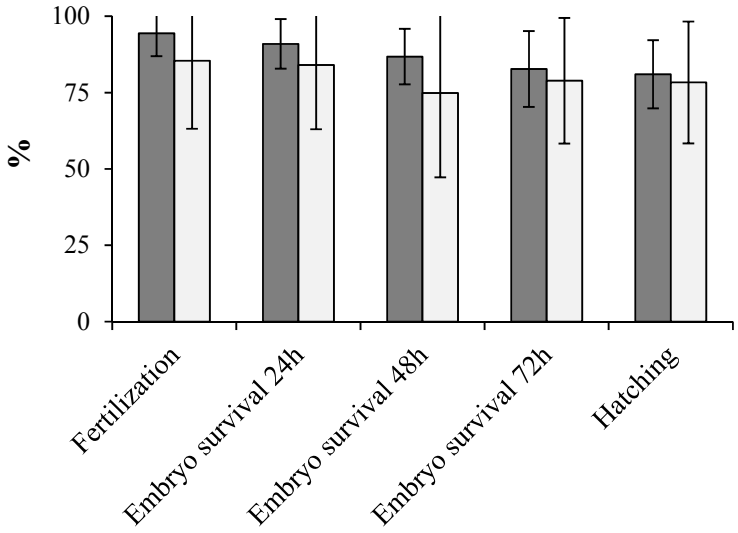


Fig. 1.5. Developmental rates (mean ± SD) of eggs of first-time (dark grey; n = 16) and successively stripped (light grey; n = 20) pikeperch spawners. Survival is not accumulated but presented for each time point. Whiskers indicate the standard deviation. No significant differences were observed.

The duration of the six spawning seasons ranged from 14 to 38 d. A high number of fish (n = 16) spawned within the first five days of the season, but after a decline from day 6 to 10 the number of spawning events increased again (Fig. 1.6). There was a significant negative correlation of the time of the season at time of stripping with the fertilization rate and embryo survival rate at 24 h (cf. table 1.3). There was no indication for difference in spawning time between first-time and successively stripped spawners (Students t-test: $F = 1.96$; $p = 0.27$) and

no relation of spawning time and fish size ($\rho = 0.17$; $p = 0.15$). The time into the spawning season was negatively correlated with *Phb2* mRNA, polar FA 15:0 and neutral 18:3(n-3), as well as ARA.

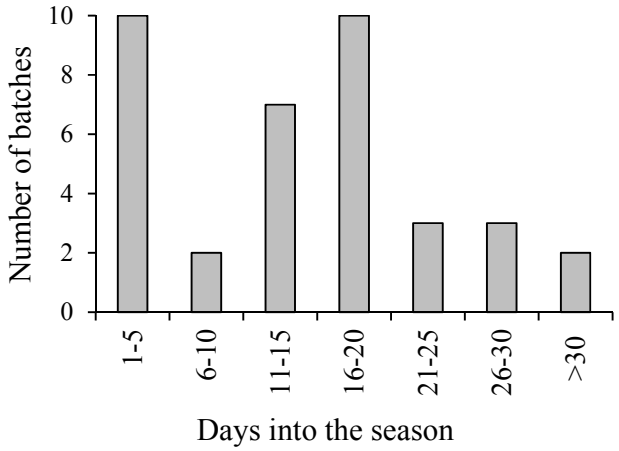


Fig. 1.6. Synchronization of spawning within the female spawners showing number of stripped females per 5 d time interval starting with day 1 after first spawning was observed in the respective season.

3.4 Prediction of egg development

For stepwise, multiple linear regression analysis respective parameters of egg composition and broodstock characteristics (incl. fecundity) were chosen, which showed significant correlations (positive or negative) with at least one indicator of egg quality. Using the parameters it was possible to explain up to 58.2% of variability in egg quality indicators (embryo survival at 24 h; Table 1.5). In fertilization rate and embryo survival at 24 h polar FA explained for significant amounts of variability. Other developmental rates could be best predicted using the fecundity, fish and egg size. Given the strong correlation between the two parameters, 88.9% of variability in the number of hatched larvae could be explained by a multiple regression including fecundity.

Table 1.5. Stepwise multiple linear regressions for all egg quality indicators with slopes of respective parameters, intercept and the total explained variability.

	Quality indicator	Fecundity (*1000 eggs)	Fish size (cm)	Egg size (mm)	Polar fatty acids			Intercept	Explained variability (%)
					15:0	18:0	20:5(n-3)*		
					($\mu g\ mg^{-1}$ dry weight)				
	Fertilization rate (%)		-1.21				17.62	145.60	47.1
Embryo survival (%)	24h		-1.11		20.73	-3.28		152.12	58.2
	48h	0.03	-0.88	-60.24				182.38	47.0
	72h	0.04	-0.80	-55.86				168.31	43.9
	Hatching rate (%)	0.04	-0.67	-59.72				161.57	46.6
	# hatched larvae	1.04	-4.64	-451.31				730.42	88.9

* EPA, eicosapentaenoic acid

4 Discussion

Variability in egg quality remains a major issue in pikeperch farming impeding a reliable supply of stocking material. Therefore, the evaluation of maternal traits, as well as current hatchery practice, which exert an influence on the biochemical composition and hence the quality of eggs is an important research topic. The main aims of this study were (I) to evaluate an array of commonly studied egg parameters associated with egg quality, (II) to determine the effect of year-round reproduction and broodstock characteristics on pikeperch egg composition and quality under commercial hatchery conditions and (III) to identify predictive biomarkers.

4.1 Egg quality parameters

4.1.1 Fatty acids

Recently, the importance of FA, especially HUFA, for broodstock nutrition, as well as embryo and larval development was reviewed in cultured freshwater percids [11, 29, 32]. In comparison to Eurasian perch [27, 28, 43], walleye [25, 44, 45] or yellow perch (*Perca flavescens*) [32], there is only limited knowledge on the FA composition in pikeperch eggs

and how this might effect reproduction and egg quality. It is well known that inter-specific differences in egg FA composition exist, even between closely related species or in-between populations [25, 44, 46]. For example, Czesny et al [45] observed high embryo viability in eggs with varying FA composition in walleye, whereas the optimal ratio of n-3/n-6 HUFA is being controversially discussed in Eurasian perch [47, 48]. Therefore, a direct comparison might be inadequate, but several aspects are certainly conserved across species, especially regarding the physiological role of HUFA [33-35].

Principally, we conclude from the overall high egg quality that the observed FA composition within the present study is close to an optimum. They generally represent characteristics of the egg FA composition of other percids (e.g., high DHA, low ARA levels) as summarized by Schaerlinger and Żarski [11]. In comparison to the report by Lund and Steenfeldt [49] on eggs of wild-caught pikeperch, we observed similarities, e.g., in total FA (here: 116.4 mg g^{-1} DW, Lund and Steenfeldt: 116.8 mg g^{-1} DW), EPA, DHA, 18:2(n-6), but there were major differences in ARA (0.8 compared to 5.5%), saturated FA (SFA; 36.1 compared to 10.7%) and monounsaturated FA (MUFA; 22.6 compared to 33.6%). In turn, Khemis et al. [46] reported even higher contents of MUFA (up to 49.8%), but similar low levels of ARA as determined in our study (1.1 – 1.5%) and again, total percentage of HUFA was lower compared to our study and the results of Lund and Steenfeldt [49]. However, both studies did not report observations on egg quality.

Here, the fraction of neutral FA was relatively large, similar to other freshwater percids [27, 44, 45]. Neutral FA are predominantly stored in the oil globule [60], but in contrast to the polar FA they are not primarily used during embryogenesis [51]. The fragmentation of the oil droplet has been suggested as an indicator of egg quality [11]. Consequently, one might expect that such fragmentation is a result of membrane composition. Therefore, it is not surprising that we detected correlations between individual polar, but not neutral FA and egg developmental rates. Here, EPA was positively correlated with fertilization and embryo survival at 24 h and an increase of this HUFA in the broodstock diet could be considered. However, the excess of FA can also have adverse effects on embryogenesis, as shown for very high n-3 HUFA in Japanese flounder (*Paralichthys olivaceus*) [52] or gilthead seabream (*Sparus aurata*) [26], resulting in lowered hatching success. In the present study, we observed such adverse effects in polar 18:0 (stearic acid, SA). Yet, it is not clear why such high polar SA levels exerted a negative influence, since the abundance was not higher compared to

previous reports in walleye eggs [45]. In bovine oocytes, the addition of SA had a negative impact on fertilization and cleavage rates [53], but there are – to our best knowledge – no reports confirming such effects in fish.

In contrast to previous observations in percid eggs [32, 44, 45], we did not detect ARA within the polar fraction of the FA. Arachidonic acid is the main precursor of eicosanoids [33-35] and is essential for embryonic development. It is not clear whether pikeperch embryos rely on the neutral ARA or elongate and desaturate 18:2(n-6) [54]. According to the results, pikeperch embryos seem to be able to cope well with such low ARA content. In other teleosts, it was shown that ARA tended to be conserved during early development [55]. Dabrowski et al. [32] observed a negative correlation of ARA with spawning time in yellow perch, which was explained by a perturbation of steroid production and resulted in lower embryo viability. This is supported by findings of Henrotte et al. [31], who detected an induction of follicle maturation in Eurasian perch in response to ARA. Though, we did not detect negative consequences of differing ARA abundance on egg quality, but high ARA was similarly correlated with early spawning. Such involvement of HUFA (especially ARA, EPA and DHA) in steroidogenesis and consequently in the control of reproduction [15, 29, 30] could also lead to increased variability of developmental success in spawners with increasing strip-spawning experience (successive stripping) as observed here by significantly lowered levels of 18:2(n-6), ARA, EPA and a higher ratio of neutral DHA/EPA (cf., below).

In general, high ratio of DHA/EPA within the neutral FA fraction could be identified as indicator of high variability in egg quality. Eggs of the most variable spawning season had significantly elevated ratio of neutral DHA/EPA, which was also negatively correlated with fertilization success and embryo survival at 24 h. However, since the neutral fraction of the FA is predominantly stored within the oil globule and not utilized during embryogenesis, we assume that such a high ratio of neutral DHA/EPA is rather an indicator of perturbation at the maternal level than a direct physiological cause of embryo mortality. Such perturbation may have been caused by an impairment of steroidogenesis and/or differing metabolism (lipid allocation) of larger fish [56]. Skalli and Robin [57] reported that dietary lipids are more likely present within the neutral fraction of the FA. Therefore, the observed relation of fecundity (and to some extent egg developmental success) and polar FA, including EPA and DHA, have possibly been a result of individual fat metabolism interlinked with endocrine perturbation. In contrast, the detected positive effects of polar EPA might be a direct effect

within the egg affecting embryo development. Eicosapentaenoic acid is involved in eicosanoid synthesis and constitutes to cell membrane bilayers [15, 56-58]. Such positive effect of EPA on egg quality has been reported also in other species [26].

The ratio of specific HUFA, has been identified as major driver of spawning quality in Eurasian perch [27] and FA are generally considered as integrative parameter being associated with egg quality. For example, it was shown that high ratios of DHA/EPA are associated with larval malformations [58]. In turn, malformations are primary causes of embryo mortality in percids [11].

4.1.2 Egg size

The ratio of neutral DHA/EPA was positively correlated with egg diameter. This may explain for the negative correlation of egg size with early embryo survival at 24 h, but not the negative effects of egg size on late embryo development (72 h) and hatching rate. The observed egg size was homogenous within clutches (average SD of 0.10 mm) and was well in the range of previous reports on pikeperch oocytes prior to fertilization and hardening [59, 60]. There are numerous studies exploring the relation of egg and maternal size, fecundity and possible implications on egg developmental success, but there is no universally valid pattern [13, 39]. Often, positive effects of egg size on the egg development have been associated with increased larval size at hatch, as well as a better nutritional state of the egg, which in turn is supposed to be beneficial for survival. However, it is being controversially discussed if egg size is generally a suitable predictive marker indicating high egg quality and observations from the field may not be transferred to hatchery conditions. Conceivably, small egg size is beneficial for survival under hatchery conditions (‘bigger is worse during incubation’ hypothesis) [61]. In chinook salmon (*Oncorhynchus tshawytscha*) a rapid evolution of small egg size could be observed in response to captivity [62]. The artificial environment during egg incubation (here: Zuger-jars) may favor smaller eggs, e.g., in terms of buoyancy or oxygen supply. Van den Berghe and Gross [63] reported similar observations with larger eggs having lower survival in poor incubation conditions in coho salmon (*Oncorhynchus kisutsch*).

4.1.3 Other parameters

Egg DW was correlated with protein content and had a negative effect on embryo survival after 24 h. Among other energy sources, embryos rely on catabolizing proteins to fuel their development [28, 40] and DW was identified as suitable marker of egg energy content [39]. Here, no positive effect of elevated DW content and only weak (positive and negative) correlations between DW and FA were observed. Consequently, we rather suggest that high DW might exert a negative influence via changes in osmoregulation, respiration in larger embryos or buoyancy of pikeperch eggs when being incubated in Zuger-jars.

Since varying cortisol levels had no effect on early development here, similar to the observations by Stratholt et al. [64] on coho salmon, cortisol cannot be directly used as biomarker for egg quality. Embryos rather seem to develop independent of observed cortisol levels. This may be due to the rapid decline of cortisol levels after fertilization, as observed in tilapia (*Oreochromis mossambicus*) [65], rainbow trout [13] and coho salmon [64], which is possibly caused by a 'loss' of cortisol during hardening. Schreck et al. [36] suggested a maternally derived protective effect from hypercortisolism. Therefore, we rather cautiously assume that high content of polar FA (incl. EPA, DHA) in the eggs is related to cortisol entry or conservation.

In contrast to our study, Bonnet et al. [38] reported a negative relation of *Phb2* with embryo development in rainbow trout. We detected correlations of *Phb2* with the majority of neutral FA, but not with rates of egg development. It could be argued that this was caused by the method applied for mRNA extraction or that mRNA storage is facilitated by high neutral FA. The physiological function of *Phb2* does not deliver clarification [66]. Principally, embryos rely on maternal provisioning of mRNA during early development [67]. However, *Phb2* does not play a role in the modulation of egg quality in pikeperch, including effects of post-ovulatory ageing. It will be necessary to further study the role of specific maternal mRNAs and their involvement in embryogenesis in a range of species, since relevance seems to differ.

4.2 Effects of maternal/broodstock characteristics on egg quality linked to biochemical egg composition

4.2.1 Year-round reproduction

In-between the six observed spawning seasons, only minor differences in egg development occurred. Surprisingly, the spring season, corresponding to the natural spawning season of pikeperch [6], showed on average higher variability, which was associated with elevated ratios of neutral DHA/EPA. Consequently, an effect of year-round production can largely be excluded as primary cause of the variation. In the course of year-round reproduction and photothermal manipulation, there seems to be no perturbation of endogenous rhythms since no substantial differences between out-off-season reproduction and the natural spawning season were observed here. These results contrast with previous reports on Eurasian perch [68] or rainbow trout where photoperiod manipulation resulted in decreased embryo survival [69]. However, the spawners sampled here were adapted to RAS conditions and photothermal manipulation.

4.2.2 Maternal size and spawning experience

Similar to the seasonal effects, the differences in fecundity can be explained by the size composition of the respective broodstocks. While fecundity was generally increasing with fish size, resulting in an overall positive rank correlation, larger fish (> 74 cm) showed decreasing number of eggs. Congruently, increased variability in egg developmental success and lower survival was already observed at smaller sizes (> 70 cm). These findings contrast sharply with the almost dogmatic believe that large, experienced spawners produce more eggs in higher quality, which is supported by numerous studies [70] and is recognized for fisheries management [71]. In freshwater percids a similar positive relation of absolute fecundity and/or egg quality with female age and/or size was observed [44, 72, 73]. In pikeperch, however, the relative fecundity does not always show a clear linear relationship with fecundity, since in some populations a decrease above ~65 cm has been reported [6]. Decreasing productivity (and higher variability in egg quality) above an optimal size can be explained by the size-dependent scaling of metabolic rates, chronic stress as a result of artificial rearing conditions, insufficient food availability or a combination of these and other

factors, as recently reviewed with emphasis on freshwater percids by Alanärä and Strand [74]. Here, fish with spawning experience were significantly larger than first-time stripped spawners. Consequently, the dominant effect of size may have masked potential consequences of successive spawning.

4.2.3 Stress and nutrition

Fish with higher energy expenses or differing lipid allocation, e.g., caused by stress [75] or individual size [76], have lower energy reserves available for reproduction. Indeed, we observed positive correlations between fecundity and – to a minor extent – maternal size and egg FA content, mainly within the polar fraction including the n-3 HUFA EPA and DHA. These FA have been associated with reproductive performance and successful larval development in several cultured fish species including pikeperch [20, 26, 32]. In rainbow trout, Watanabe et al. [77] reported a decrease in fecundity and an increased mortality of early stages in response to n-3 HUFA deficiency. Here, females within a respective size range of ~65 to 74 cm produced high numbers of eggs with high levels of specific polar FA indicating optimal conditions.

In parallel, cortisol levels were negatively correlated with fecundity and FA content and declined with increasing fish size. Cortisol levels observed here are well in the range of previous reports on plasma cortisol levels of pikeperch spawners [78]. This pattern suggests a direct influence of stress on fecundity, which is expressed via the FA deposition. There is growing evidence related to the inter-linkage of stress and FA, especially HUFA [21, 22, 79]. However, we found that large fish with decreasing fecundity had similar cortisol levels compared to highly productive intermediate sized fish. In small fish elevated cortisol levels had no negative effect on egg developmental success. Therefore, such a direct mediation of handling or rearing stress on fecundity seems rather unlikely. Campbell et al. [80, 81] observed negative effects of handling stress on embryo and larval survival, but not on fecundity in trout species. Here, decreased contents of specific FA in oocytes with high cortisol levels indicate a stress-mediated lack of maternal provisioning of FA, which in turn affected fertilization and embryo survival.

Nutrition is of utmost importance for reproduction affecting spawner condition and subsequent nutrient mobilization, as well as processes involved in the endocrine control of

reproduction, e.g., by the actions of the adipocyte derived hormone leptin on the expression of gonadotropin releasing hormone [82]. Feed restriction has been associated with decreasing fecundity and egg quality in other teleost species [15, 83]. Here, fish were fed *ad libitum* rations. Still, fish with increasing strip-spawning experience could potentially not (re)build sufficient nutrient reserves in-between spawning events. This could explain the observed differences in FA composition (polar 18:2(n-6), ARA, EPA, neutral DHA/EPA ratio) between fish with differing spawning history. However, we can only draw cautious conclusions from our results on the relation of female condition, stress and the observed patterns in fecundity, egg quality and FA acid composition and this topic certainly requires further research.

4.2.4 Spawning time

Fertilization and embryo survival (24 h) declined over time of the season, but it was still possible to obtain high quality eggs from individual females spawning relatively late (day 29 and 31). For example, the overall highest number of hatched larvae (760,200) was observed in a late-spawning female (day 31). It can be argued that decreasing sperm quality with season length, as observed in other species [84, 85], had a negative impact on fertilization success, but high fertilization rates (99.0%) could occasionally be observed towards the end of the season. Therefore, the assumed benefits of early spawning cannot be generalized.

4.3 Post-ovulatory ageing

In general, the egg components observed here did not change in aged eggs. Post-ovulatory ageing has been identified as a major issue regarding the *in vitro* fertilization of fish [12, 14, 16] due to the difficulties related to the correct timing of stripping [60]. In pikeperch, post-ovulatory ageing was associated with increased mitochondrial DNA damage, as well as lowered antioxidant capacity [28] and higher incidence of triploidization [86]. Within the three aged clutches, only eggs of one female could be fertilized but the entire batch was lost within the first 24 h. These findings contrast with observations by Samarin et al. [86], who reported no effect of post-ovulatory ageing (up to 18 h) on fertilization and hatching rates of pikeperch eggs. Therefore, the effect of ‘over-ripening’ in this species remains controversial.

4.4 Egg development and predictive quality parameters

The developmental stages considered to assess egg developmental potential in this study are relatively easy to access and less susceptible towards external parameters, compared to post-hatch embryos and larvae. In addition to intrinsic factors larvae are exposed to a variety of extrinsic factors after hatching. These factors modulate development and survival, such as external feeding, light and temperature, stocking densities or cannibalism [87]. These influences potentially interfere with the initial, inherent quality of the egg and herewith exacerbate the comparability. It can be assumed that many parameters observed here, e.g., neutral FA, exert an influence on the quality of post-hatch larvae. For example, it was shown that the stress response of pikeperch larvae differs depending on the HUFA tissue content [49].

No specific stage (24, 48, 72 h survival or hatching rate) with significantly elevated mortality could be identified here. However, the largest differences were observed between fertilization and 24 h (3.5% mortality) and again between 24 and 48 h (6.3% mortality) indicating a more drastic mortality within this period of time. Schaerlinger and Żarski [11] reported high mortality in Eurasian perch during the first 24 h possibly caused by cleavage defects or impairment of genome activation. Here, only 1.5% of embryos, which survived until 48 h, did not hatch. The majority of significant correlations between egg development and observed parameters (FA, time into the season, DW content, egg size) could be detected until 24 h. Consequently, using multiple linear regression analysis the highest amount of explained variability (58.2%) was observed at embryo survival during this period. These results highlight that embryos are highly dependent on the biochemical composition of the oocytes in particular during early embryogenesis. With progressing development, embryos gain increasing independence of the inherent egg composition

Conclusions

We observed overall relatively high egg quality underlining the effectiveness of the applied hatchery protocol. Still, there was a substantial variability in fecundity and – to a certain extent – egg and embryo development revealing potential for optimization and fine-tuning of reproductive management (spawner selection and broodstock rearing). In contrast to our hypothesis, increased variability was not caused by effects of out-of-season reproduction

and herewith endogenous zeitgeber of reproduction, but rather by maternal traits. Potential perturbations at the maternal level, such as increasing spawning history and stress, were reflected in the biochemical composition of the eggs, especially in HUFA content, which – in turn – was correlated with fecundity and developmental success. Therefore, year-round reproduction has no effect on egg quality, especially when using spawners within an optimal size range of ~65 – 70 cm. Female length could be identified as major determinant of egg quality. Substantial amounts of variability in egg developmental success could be explained by a combination of egg parameters and maternal traits highlighting the importance of the biochemical egg composition especially during the early embryogenesis when mortality is highest. Consequently, we suggest the development of species-specific broodstock diets based on our findings. The selection of breeders is critical to maximize and stabilize larvae production. Therefore, reproductive performance of large individuals has to be carefully monitored, as well as possible size-selective processes during egg incubation. Due to the detected links between maternal characteristics and biochemical egg composition, a broad range of parameters and their manipulation, e.g., via nutrition, has to be explored to further understand these interactions, as well as to improve production of pikeperch.

Acknowledgements

We would like to thank our cooperation partner AquaPri and the members of the IGB working group Sustainable Aquaculture and Applied Physiology, especially E. Kreuz, as well as V. Schoening for the support in the lab.

References

1. FAO. The State of World Fisheries and Aquaculture 2014. Rome, 243 pp.
2. European Commission. Strategic Guidelines for the sustainable development of EU aquaculture. COM(2013) 229. Brussels, 29.4.2013.
3. Fontaine P, Wang N, Hermelink B. Broodstock Management and Control of the Reproductive Cycle. In: Kestemont P, Dabrowski K, Summerfelt RC (eds.), Biology and Culture of Percid Fishes. Dordrecht: Springer Verlag; 2015:103-122.
4. Mylonas CC, Fostier A, Zanuy S. Broodstock management and hormonal manipulations of fish reproduction. Gen Comp Endocrinol 2010; 165:516-534.
5. Taranger GL, Carrillo M, Schulz RW, Fontaine P, Zanuy S, Felip A, Weltzien FA, Dufour S, Karlsen Ø, Norberg B, Andersson E, Hansen T. Control of puberty in farmed fish. Gen Comp Endocrinol 2010; 165: 483-515.
6. Lappalainen J, Dörner H, Wysujack K. Reproduction biology of pikeperch (*Sander lucioperca* (L.)) - a review. Ecol Freshw Fish 2003; 12:95-106.
7. Żarski D, Horváth Á, Held JA, Kucharczyk D. Artificial Reproduction of Percid Fishes. In: Kestemont P, Dabrowski K, Summerfelt RC (eds.), Biology and Culture of Percid Fishes. Dordrecht: Springer Verlag; 2015:123-162.
8. Mueller-Belecke A, Zienert S. Out-of-season spawning of pike perch (*Sander lucicoperca* L.) without the need for hormonal treatments. Aquacult Res 2008; 39:1279-1285.
9. Zakęś Z, Demska-Zakęś K. Controlled reproduction of pikeperch *Sander lucioperca* (L.): a review. Arch Pol Fish 2005; 17:153-170.
10. Overton JL, Toner D, Poliçar T, Kucharczyk D. Commercial Production: Factors for Success and Limitations in European Percid Fish Culture. In: Kestemont P, Dabrowski K, Summerfelt RC (eds.), Biology and Culture of Percid Fishes. Dordrecht: Springer Verlag; 2015:881-890.
11. Schaerlinger B, Żarski D. Evaluation and Improvements of Egg and Larval Quality in Percid Fishes. In: Kestemont P, Dabrowski K, Summerfelt RC (eds.), Biology and Culture of Percid Fishes. Dordrecht: Springer Verlag; 2015:193-226.
12. Bobe J, Labbé C. Egg and sperm quality in fish. Gen Comp Endocrinol 2010; 165:535-548.

13. Brooks S, Tyler CR, Sumpter JP. Quality in fish: what makes a good egg? Rev Fish Biol Fish 1997; 7:387-416.
14. Migaud H, Bell G, Cabrita E, McAndrew B, Davie A, Bobe J, Herraez MP, Carrillo M. Gamete quality and broodstock management in temperate fish. Rev Aquacult 2013; 5:194-223.
15. Izquierdo MS, Fernández-Palacios H, Tacon AGJ. Effect of broodstock nutrition on reproductive performance of fish. Aquaculture 2001; 197:25-42.
16. Valdebenito II, Gallegos PC, Effer BR. Gamete quality in fish: evaluation parameters and determining factors. Zygote 2015; 23:177-197.
17. Hermelink B, Wuertz S, Trubiroha A, Rennert B, Kloas W, Schulz C. Influence of temperature on puberty and maturation of pikeperch, *Sander lucioperca*. Gen Comp Endocrinol 2011; 172:282-292.
18. Hermelink B, Wuertz S, Rennert B, Kloas W, Schulz C. Temperature control of pikeperch (*Sander lucioperca*) maturation in recirculating aquaculture systems-induction of puberty and course of gametogenesis. Aquaculture 2013; 400:36-45.
19. McCormick MI. Behaviorally induced maternal stress in a fish influences progeny quality by a hormonal mechanism. Ecology 1998; 79:1873-1883.
20. Lund I, Skov PV, Hansen BW. Dietary supplementation of essential fatty acids in larval pikeperch (*Sander lucioperca*); short and long term effects on stress tolerance and metabolic physiology. Comp Biochem Physiol 2012; 162:340-348.
21. Moodie GEE, Loadman NL, Wiegand MD, Mathias JA. Influence of egg characteristics on survival, growth and feeding in larval walleye (*Stizostedion vitreum*). Can J Fish AquatSci 1989; 46:516-521.
22. Van Anholt RD, Spanings EAT, Koven WM, Nixon O, Bonga SEW. Arachidonic acid reduces the stress response of gilthead seabream *Sparus aurata* L. J ExpBiol 2004; 207:3419-3430.
23. Cerdá J, Zanuy S, Carrillo M, Ramos J, Serrano R. Short-and long-term dietary effects on female sea bass (*Dicentrarchus labrax*): seasonal changes in plasma profiles of lipids and sex steroids in relation to reproduction. Comp Biochem Physiol 1995; 111:83-91.
24. Mercure F, Van Der Kraak G. Mechanisms of action of free arachidonic acid on ovarian steroid production in the goldfish. Gen Comp Endocrinol 1996; 102:130-140.

25. Czesny S, Dabrowski K. The effect of egg fatty acid concentrations on embryo viability in wild and domesticated walleye (*Stizostedion vitreum*). Aquat Living Resour 1998; 11:371-378.
26. Fernández-Palacios H, Izquierdo MS, Robaina L, Valencia A, Salhi M, Vergara JM. Effect of n− 3 HUFA level in broodstock diets on egg quality of gilthead sea bream (*Sparus aurata* L.). Aquaculture 1995; 132:325-337.
27. Henrotte E, Mandiki RSNM, Prudencio AT, Vandecan M, Mélard C, Kestemont P. Egg and larval quality, and egg fatty acid composition of Eurasian perch breeders (*Perca fluviatilis*) fed different dietary DHA/EPA/AA ratios. Aquacult Res 2010; 41:53-61.
28. Abi-Ayad S, Kestemont P, Mélard C. Dynamics of total lipids and fatty acids during embryogenesis and larval development of Eurasian perch (*Perca fluviatilis*). Fish Physiol Biochem 2000; 23:233-243.
29. Kestemont P, Henrotte E. Nutritional Requirements and Feeding of Broodstock and Early Life Stages of Eurasian Perch and Pikeperch. In: Kestemont P, Dabrowski K, Summerfelt RC (eds.), Biology and Culture of Percid Fishes. Dordrecht: Springer Verlag; 2015:539-564.
30. Navas JM, Mañanós E, Thrush M, Ramos J, Zanuy S, Carrillo M, Zohar Y, Bromage N. Effect of dietary lipid composition on vitellogenin, 17 beta-estradiol and gonadotropin plasma levels and spawning performance in captive sea bass (*Dicentrarchus labrax* L). Aquaculture 1998; 165:65-79.
31. Henrotte E, Milla S, Mandiki SNM, Kestemont P. Arachidonic acid induces production of 17, 20β-dihydroxy-4-pregnen-3-one (DHP) via a putative PGE2 receptor in fish follicles from the Eurasian perch. Lipids 2011; 46:179-187.
32. Dabrowski K, Rinchard J, Czesny S, Korzeniowska M. Effects of Dietary Levels of PUFA Fed to Adult Yellow Perch on the Fatty Acid Composition of Eggs and Larvae Characteristics: New Research Directions. In: Kestemont P, Dabrowski K, Summerfelt RC (eds.), Biology and Culture of Percid Fishes. Dordrecht: Springer Verlag; 2015:565-586.
33. Bell MV, Henderson RJ, Sargent JR. The role of polyunsaturated fatty acids in fish. Comp Biochem Physiol 1986; 83:711-719.
34. Sargent JR, Tocher DR, Bell JG. The lipids. In: Halver JE, Hardy RW (eds.), Fish Nutrition, 3rd ed. San Diego: Academic press; 2002:181–257.

35. Tocher DR. Metabolism and functions of lipids and fatty acids in teleost fish. Rev Fish Sci 2003; 11:107-184.
36. Schreck CB, Contreras-Sanchez W, Fitzpatrick MS. Effects of stress on fish reproduction, gamete quality, and progeny. Aquaculture 2001; 197:3-24.
37. Schaefer FJ, Overton JL, Wuertz S. Pikeperch *Sander lucioperca* egg quality cannot be predicted by total antioxidant capacity and mtDNA fragmentation. Anim Reprod Science 2016; 167:117-124.
38. Bonnet E, Fostier A, Bobe J. Microarray-based analysis of fish egg quality after natural or controlled ovulation. Bmc Genomics 2007; 8.
39. Kamler E. Parent-egg-progeny relationships in teleost fishes: an energetics perspective. Rev Fish Biol Fish 2005; 15 :399-421.
40. Jobling M. Fish bioenergetics. London: Chapman & Hall; 1994.
41. Boëchat IG, Krüger A, Chaves RC, Graeber D, Gücker B. Land-use impacts on fatty acid profiles of suspended particulate organic matter along a larger tropical river. Sci Total Environ 2014; 482:62-70.
42. Kroupova H, Trubiroha A, Wuertz S, Kloas W. Stage-dependent differences in RNA composition and content affect the outcome of expression profiling in roach (*Rutilus rutilus*) ovary. Comp Biochem Physiol 2010; 159:141-149.
43. Żarski D, Krejszeff S, Horváth Á, Bokor Ż, Palińska K, Szentes K., Łuczyńska J, Targońska K, Kupren K, Urbányi B, Kucharczyk D. Dynamics of composition and morphology in oocytes of Eurasian perch, *Perca fluviatilis* L., during induced spawning. Aquaculture 2012; 364:103-110.
44. Wiegand MD, Johnston TA, Martin J, Leggett WC. Variation in neutral and polar lipid compositions of ova in ten reproductively isolated populations of walleye (*Sander vitreus*). Can J Fish Aquat Sci 2004; 61:110-121.
45. Czesny S, Rinchard J, Dabrowski K. Intrapopulation variation in egg lipid and fatty acid composition and embryo viability in a naturally spawning walleye population from an inland reservoir. N Am J Fish Manage 2005; 25:122-129.
46. Khemis IB, Hamza N, Messaoud NB, Rached SB, M'Hetli M. Comparative study of pikeperch *Sander lucioperca* (Percidae; Linnaeus, 1758) eggs and larvae from wild females or from captive females fed chopped marine fish. Fish Physiol Biochem 2014; 40:375-384.

47. Blanchard G, Druart X, Kestemont P. Lipid content and fatty acid composition of target tissues in wild *Perca fluviatilis* females in relation to hepatic status and gonad maturation. J Fish Biol 2005; 66:73-85.
48. Henrotte E, Overton JL, Kestemont P. Effects of dietary n-3 and n-6 fatty acids levels on egg and larval quality of Eurasian perch. Cybium 2008; 32:271-272.
49. Lund I, Steenfeldt SJ. The effects of dietary long-chain essential fatty acids on growth and stress tolerance in pikeperch larvae (*Sander lucioperca* L.). Aquacult Nutr 2011; 17:191-199.
50. Moodie GEE, Loadman NL, Wiegand MD, Mathias JA. Influence of egg characteristics on survival, growth and feeding in larval walleye (*Stizostedion vitreum*). Can J Fish Aquat Sci 1989; 46:516-521.
51. Wiegand MD. Composition, accumulation and utilization of yolk lipids in teleost fish. Rev Fish Biol Fish 1996; 6:259-286.
52. Furuita H, Tanaka H, Yamamoto T, Suzuki N, Takeuchi T. Effects of high levels of n-3 HUFA in broodstock diet on egg quality and egg fatty acid composition of Japanese flounder, *Paralichthys olivaceus*. Aquaculture 2002; 210:323-333.
53. Leroy J, Vanholder T, Mateusen B, Christophe A, Opsomer G, de Kruif A, Genicot G, Van Soom A. Non-esterified fatty acids in follicular fluid of dairy cows and their effect on developmental capacity of bovine oocytes in vitro. Reproduction 2005; 130:485-495.
54. Tocher DR. Omega-3 long-chain polyunsaturated fatty acids and aquaculture in perspective. Aquaculture 2015; 449:94-107.
55. Gunasekera RM, De Silva SS, Ingram BA. Early ontogeny-related changes of the fatty acid composition in the Percichthyid fishes trout cod, *Maccullochellam acquariensis* and Murray cod, *M. peeliipeelii.* Aquat Living Resour 199; 12:219-227.
56. Jobling M. Nutrient partitioning and the influence of feed composition on body composition. In: Houlihan D, Boujard T, Jobling M (eds.), Food intake in fish. Oxford: Blackwell; 2001:354-375.
57. Skalli A, Robin JH. Requirement of n-3 long chain polyunsaturated fatty acids for European sea bass (*Dicentrarchus labrax*) juveniles: growth and fatty acid composition. Aquaculture 2004; 240:399-415.

58. Yang Q, Zheng P, Ma Z, Li T, Jiang S, Qin JG. Molecular cloning and expression analysis of the retinoid X receptor (RXR) gene in golden pompano *Trachinotus ovatus* fed Artemia nauplii with different enrichments. Fish Physiol Biochem 2015; 41:1449-1461.
59. Demska-Zakęś K, Zakęś Z, Roszuk J. The use of tannic acid to remove adhesiveness from pikeperch, *Sander lucioperca*, eggs. Aquacult Res 2005; 36:1458-1464.
60. Żarski D, Kucharczyk D, Targońska K, Palińska K, Kupren K, Fontaine P, Kestemont P. A new classification of pre-ovulatory oocyte maturation stages in pikeperch, *Sander lucioperca* (L.), and its application during artificial reproduction. Aquacult Res 2012; 43:713-721.
61. Einum S, Hendry AP, Fleming IA. Egg-size evolution in aquatic environments: does oxygen availability constrain size? Proc R Soc B 2002; 269:2325-2330.
62. Heath DD, Heath JW, Bryden CA, Johnson RM, Fox CW. Rapid evolution of egg size in captive salmon. Science 2003; 299:1738-1740.
63. Van den Berghe EP, Gross MR. Natural selection resulting from female breeding competition in a Pacific salmon (coho: *Oncorhynchus kisutch*). Evolution 1989; 43:125-140.
64. Stratholt ML, Donaldson EM, Liley NR. Stress induced elevation of plasma cortisol in adult female coho salmon (*Oncorhynchus kisutch*), is reflected in egg cortisol content, but does not appear to affect early development. Aquaculture 1997; 158:141-153.
65. Hwang PP, Wu SM, Lin JH, Wu LS. Cortisol content of eggs and larvae of teleosts. Gen Comp Endocrinol 1992; 86:189-196.
66. Ikonen E, Fiedler K, Parton RG, Simons K.. Prohibitin, an antiproliferative protein, is localized to mitochondria. FEBS Letters. 1995; 358:273-277.
67. Dworkin MB, Dworkin-Rastl E. Functions of Maternal Messenger-RNA in Early Development. MolReprod Dev 1990; 26:261-297.
68. Żarski D, Palińska K, Targońska K, Bokor Z, Kotrik L, Krejszeff S, Kupren K, Horváth Á, Urbányi B, Kucharczyk D. Oocyte quality indicators in Eurasian perch, *Perca fluviatilis* L., during reproduction under controlled conditions. Aquaculture 2011; 313:84-91.
69. Bonnet E, Fostier A, Bobe J. Characterization of rainbow trout egg quality: A case study using four different breeding protocols, with emphasis on the incidence of embryonic malformations. Theriogenology 2007; 67:786-794.

70. Feiner ZS, Höök TO. Environmental Biology of Percid Fishes. In: Kestemont P, Dabrowski K, Summerfelt RC (eds.), Biology and Culture of Percid Fishes. Dordrecht: Springer Verlag; 2015:61-100.
71. Birkeland C, Dayton PK. The importance in fishery management of leaving the big ones. Trends Ecol Evol 2005; 20:356-358.
72. Johnston TA, Lysack W, Leggett WC. Abundance, growth, and life history characteristics of sympatric walleye (*Sander vitreus*) and sauger (*Sander canadensis*) in Lake Winnipeg, Manitoba. J Great Lakes Res 2012; 38:35-46.
73. Żivkov M, Petrova G. On the pattern of correlation between the fecundity, length, weight and age of pikeperch *Stizostedion lucioperca*. J Fish Biol 1993; 43:173-182.
74. Alanärä A, Strand Å. The Energy Requirements of Percid Fish in Culture. In: Kestemont P, Dabrowski K, Summerfelt RC (eds.), Biology and Culture of Percid Fishes. Dordrecht: Springer Verlag; 2015:353-368.
75. Strand Å, Magnhagen C, Alanärä A. Effects of repeated disturbances on feed intake, growth rates and energy expenditures of juvenile perch, *Perca fluviatilis*. Aquaculture 2007; 265:163-168.
76. Strand Å, Overton JL, Alanärä A. The energy requirements of Eurasian perch (*Perca fluviatilis*L.) in intensive culture. Aquacult Nutr 2011; 17:818-824.
77. Watanabe T, Takeuchi T, Saito M, Nishimura K. Effect of low protein-high calory or essential fatty acid deficiency diet on reproduction of rainbow trout. Bull Jpn Soc Sci Fish 1984; 50:1207–1215.
78. Sarameh SP, Falahatkar B, Takami GA, Efatpanah I. Physiological changes in male and female pikeperch *Sander lucioperca* (Linnaeus, 1758) subjected to different photoperiods and handling stress during the reproductive season. Fish Physiol Biochem 2013; 39:1253-1266.
79. Martins DA, Rocha F, Castanheira F, Mendes A, Pousão-Ferreira P, Bandarra N, Coutinho J, Morais S, Yúfera M, Conceição LEC, Martínez-Rodriguez G. Effects of dietary arachidonic acid on cortisol production and gene expression in stress response in Senegalese sole (*Solea senegalensis*) post-larvae. Fish Physiol Biochem 2013; 39:1223-1238.
80. Campbell PM, Pottinger TG, Sumpter JP. Stress reduces the quality of gametes produced by rainbow trout. Biol Reprod 1992; 47:1140-1150.

81. Campbell PM, Pottinger TG, Sumpter JP. Preliminary evidence that chronic confinement stress reduces the quality of gametes produced by brown and rainbow trout. Aquaculture 1994; 120:151-169.
82. Oakley AE, Clifton DK, Steiner RA. Kisspeptin Signaling in the Brain. Endocr Rev 2009; 30:713-743.
83. Kjørsvik E, Mangorjensen A, Holmefjord I. Egg Quality in Fishes. Adv Mar Biol 1990; 26:71-113.
84.Alavi SMH, Rodina M, Hatef A, Stejskal V, Poliçar T, Hamackova J, Linhart O. Sperm motility and monthly variations of semen characteristics in *Perca fluviatilis* (Teleostei: Percidae). Czech J Anim Sci 2010; 55:174-182.
85. Król J, Glogowski J, Demska-Zakęś K, Hliwa P. Quality of semen and histological analysis of testes in Eurasian perch *Perca fluviatilis* L. during a spawning period. Czech J Anim Sci 2006; 51:220-226.
86. Samarin AM, Blecha M, Bytyutskyy D, Poliçar T. Post-Ovulatory Oocyte Ageing in Pikeperch (*Sander lucioperca* L.) and its Effect on Egg Viability Rates and the Occurrence of Larval Malformations and Ploidy Anomalies. Turk J Fish Aquat Sci 2015; 15:435-441.
87. Steenfeldt S. Culture Methods of Pikeperch Early Life Stages. In: Kestemont P, Dabrowski K, Summerfelt RC (eds.), Biology and Culture of Percid Fishes. Dordrecht: Springer Verlag; 2015:295-312.

Chapter II

Pikeperch *Sander lucioperca* egg quality cannot be predicted by total antioxidant capacity and mtDNA fragmentation

Pikeperch *Sander lucioperca* egg quality cannot be predicted by total antioxidant capacity and mtDNA fragmentation

Fabian J. Schaefer, Julia L. Overton and Sven Wuertz

Animal Reproduction Science, DOI: 10.1016/j.anireprosci.2016.02.016

Abstract

In farmed pikeperch, there is a high variability in egg quality restraining the propagation of this species in aquaculture. The identification of reliable biomarkers for predicting successful embryo development already at an early stage (unfertilized oocyte) could help improve production efficiency. Total antioxidant capacity (TAC) and the quantification of mitochondrial DNA (mtDNA) fragmentation have been established as biomarkers for oxidative stress and damage of macromolecules, potentially influencing embryo development. Therefore, we evaluated these biomarkers in eggs of commercially farmed pikeperch (44 females). We measured egg TAC, as well as lesion rates per 10 kb of 12S and cytochrome b (cytb) as target regions within the mitochondrial genome by qPCR. It was tested whether these markers correlate with embryo development (fertilization rate, embryo survival, hatching rate). There was no significant relation of mtDNA lesion rates or TAC with these egg quality parameters. We detected average lesion rates (±SD) of 1.50 (±1.57) and 1.89 (±2.14) in 12S and cytb mtDNA respectively. Lesion rates in 12S and cytb were highly correlated within samples ($P < 0.0001$) and were independent of the observed TAC. The results suggest that TAC does not prevent mtDNA fragmentation and that embryos rather seem to be able to cope with the observed fragmentation of mtDNA. However, in post-ovulatory aged eggs of three females with little to no fertilization success, lesion rates of cytb were significantly elevated, whereas TAC was significantly lower compared to other females, suggesting a possible role of oxidative stress during post-ovulatory ageing.

1 Introduction

Aquaculture is one of the fastest growing sectors of food production worldwide (FAO, 2014). To increase species diversification in this sector, new candidate fish species are introduced to farming to meet market demands. However, to overcome the candidate status of new species towards domestication, the reliable production of high quality gametes is a major prerequisite (Migaud et al., 2013). Only the constant availability of stocking material allows for an up-scaling of production. In intensive aquaculture, reproduction of fish can be achieved e.g., through photothermal induction of maturation with or without the use of stimulating hormones (cf. Donaldson, 1996 and Mylonas et al., 2010 for review). As in wild fish, such controlled induction of reproduction results in strong inter-individual variability of gamete quality (Brooks et al., 1997; Bobe and Labbé, 2010). As a consequence, production costs remain on a high level, because broodstock and hatchery capacities need to be large enough to cope with losses caused by low biological quality. Accordingly, the identification of female and/or batch-specific biomarkers, which can reliably predict future developmental success of offspring remains a major task (Bobe and Labbé, 2010; Migaud et al., 2013). Understanding the underlying biochemical and molecular drivers of gamete quality can potentially improve reproductive performance and hence optimize the reproductive management of species in aquaculture (e.g., via food additives or gamete handling procedures).

Oxidative stress, the imbalance of reactive oxygen species (ROS) and inherent defense mechanisms, has been identified as potential cause affecting cell functioning and early embryo development (Aitken and Baker, 2004; Dennery, 2007; Metcalfe and Alonso-Alvarez, 2010) and analyses dealing with this fragile balance could be used as biomarkers of gamete quality. Oxidative stress can cause damage of essential genetic information, such as DNA strand breaks and base modifications especially in mitochondria, which exhibit high chronic ROS exposure (Yakes and van Houten, 1997; Rothfuss et al., 2010). Therefore, an egg with impaired antioxidant status is likely to exhibit abnormal development. There are two hierarchical levels affecting oxidative stress where the application of biomarkers seems most useful: the inherent defense mechanisms against oxidative stress and the damage caused as consequence of oxidative stress.

The mitochondrial DNA (mtDNA) is an ideal target for detecting ROS-induced damage in eggs. First, mtDNA is presumably more prone to damage compared to nuclear DNA

(Cartón-García et al., 2013; Sawyer et al., 2003; Yakes and van Houten, 1997). Second, in most cases the entirety of mitochondria is inherited maternally, so paternal influences can – to a large extent – be excluded (Schwartz and Vissing, 2002; Wolff and Gemmell, 2008) resulting in maternal (egg) specific markers. In addition, the mitochondrial genome is present in a high copy number in each fish oocyte, which allows for a representative assessment (Artuso et al., 2012). However, the majority of methods for detecting DNA fragmentation (e.g., comet assays) are relatively unspecific, only partially quantitative and do not allow a separated analysis of mtDNA and nDNA damage (Tice et al., 2000). A new sensitive method to quantify DNA lesion rates has been recently developed (Rothfuss et al., 2010). The method utilizes the quantification of a short and a long fragment of a specific target region by qPCR for the quantification of DNA lesions (Rothfuss et al., 2010). This method has successfully been applied to detect DNA lesions caused by cryopreservation of zebrafish *Danio rerio* primordial germ cells (Riesco and Robles, 2012) and gilt-head bream *Sparus aurata* sperm (Cartón-García et al., 2013). However, the method has not yet been applied for the use as a biomarker for the assessment of egg quality in fish and knowledge about the physiological consequences of mtDNA lesions remains vague.

The determination of total antioxidant capacity (TAC) has previously been used as proxy for sperm quality (Mahfouz et al., 2009; Gürler et al., 2015) and is generally regarded as potent marker of oxidative stress (Ghiselli et al., 2000; Kusano and Ferrari, 2008), but to our best knowledge, it has not yet been used as biomarker for egg quality in fish. Measurement of TAC can potentially be a valuable determinant of egg quality, since several studies have shown that certain antioxidants, such as α-tocopherol and vitamin C, are beneficial, if not crucial for embryonic development in fish (cf. review by Izquierdo et al., 2001).

The main aim of the present study was the evaluation of the applicability of mtDNA damage and TAC as biomarkers for the prediction of egg quality in pikeperch *Sander lucioperca* under hatchery conditions. Over the course of two consecutive years, a total of 44 unfertilized egg batches from pikeperch broodstock were sampled on a commercial farm and fertilization, survival of embryos (after 24, 42 and 72h) and hatching rate were assessed as estimates of egg quality. In addition to TAC, lesion rates in two target regions of the mitochondrial genome encoding for 12S and cytochrome b (cytb) were determined by qPCR. We hypothesized that elevated TAC prevents mtDNA lesions. Further, we expected high numbers of mtDNA lesions to negatively influence embryo development, whereas elevated

TAC levels are expected to be beneficial. This study contributes to understanding the consequential effects of oxidative stress and inherent protection through antioxidants during embryogenesis. By identifying inter-individual differences in these parameters, broodstock composition as well as reproductive management may be optimized in commercial aquaculture of pikeperch.

2 Material and methods

2.1 Sampling

Ovulated eggs were collected from a total of 44 female pikeperch over two consecutive years at a commercial aquaculture facility (AquaPri, Denmark). Broodstock maturation was induced by wintering below 14 °C for three months and subsequent warming to ~16 °C to trigger ovulation. No hormone treatment was used. Broodstock fish of both sexes were reared together.

At time of ovulation, female fish were anesthetized (Kalmagin 20%, Centrovet, Santiago, Chile) and eggs were stripped. A subset of eggs from each of the females was immediately frozen at -20 °C and transported to the IGB Berlin in liquid nitrogen for further storage at -80 °C. For the majority of females (n = 33), the remaining eggs were fertilized with freshly stripped sperm and transferred into Zuger-jars until hatching. A minimum of 50 eggs were considered at the designated time points in triplicates to determine fertilization rate (%), survival after 24, 48, 72 h (%) and hatching rate (%) on day four post fertilization by use of a microscope (Stereozoom IT-TR, Gundlach, Marburg, Germany). In some cases, not all five quality parameters could be assessed, due to rearing limitations or hatchery related operations.

2.2 DNA extraction and fragmentation standards

For each sample ~30 mg (wet weight) of eggs were extracted using a commercial kit (peqGOLD Tissue DNA Mini Kit; PeqLab, Erlangen, Germany). The concentration of double-stranded DNA (ng μl^{-1}) was measured by UV absorption spectrometry (Nanodrop ND-1000 spectrophotometer; Thermo Fisher Scientific, Waltham, MA, USA). Total DNA was subsequently diluted to 7 ng μl^{-1}. Extraction of high molecular weight DNA was confirmed by electrophoresis.

A sub-sample of eggs from one random female was divided into four portions (~30 mg egg wet weight portion^{-1}). One portion was not further treated (control). The other portions were placed under UV light (302 nm) for 5, 10 and 20 min respectively, to generate graduated DNA fragmentation. The DNA of these four egg portions was extracted, measured and diluted accordingly.

2.3 Assessment of lesions by qPCR

For both 12S and cytb targets, qPCR assays (Table 2.1) for a long (l) and a short (s) fragment were established based on sequence information available (NCBI accession No KP125333). Specific amplification was confirmed by direct sequencing (SeqLab, Göttingen, Germany) and subsequent sequence analysis. All qPCR reactions were determined in duplicate in a Mx3005 cycler (Agilent, Santa Clara, CA, USA) using the same temperature protocol [10 min initial degeneration at 96 °C, followed by 40 cycles of degeneration at 96 °C, annealing at 60 °C and elongation at 72 °C]. Product specificity was confirmed by direct sequencing and checked for each reaction by melting-curve analysis. Amplification was carried out using hot start polymerase and SYBR Green I (Qiagen, Venlo, Netherlands) as fluorescent dye in a 25 µL reaction volume [14 ng DNA, 0.4 µM of each primer, 1x Taq buffer, 3.5 mM (s12S, l12S) or 5 mM (scytb, lcytb) $MgCl_2$, 0.2 mM of each dNTP, 0.125 µL of 100-fold diluted SYBR-Green I solution, 4 U (s12S, l12S) or 2U (scytb) Platinum Taq or 2 U Phire II Taq (lcytb) (Thermo Fisher Scientific)]. Fragmentation of DNA of both targets (12S and cytb) was calculated according to Rothfuss et al. (2010). The difference in threshold cycles of the long and the short fragment of the target is used to calculate the lesion rate per 10 kb of mtDNA:

$$\text{Lesions per 10 kb} = (1 - 2^{-(\Delta \text{ long fragment} - \Delta \text{ short fragment})}) \times 10{,}000 \text{ [bp]} / \text{size of long fragment [bp]}$$

2.4 Total antioxidant capacity (TAC)

The TAC (mM Trolox equivalent) was determined using a commercial antioxidant assay (Sigma-Aldrich, St. Louis, MO, USA). Absorbance was measured at a wavelength of 570 nm with an Infinite M200 PRO microplate reader (Tecan, Männerdorf, Switzerland). For each batch, ~50 mg of frozen eggs were manually crushed with a pistil in 1 ml of water. Upon centrifugation (5 min at 9,140 g, 4 °C), 5 µl of the supernatant was analyzed in duplicate

according to the manual. Individual high TAC measurements exceeding the standard curve were repeated after a 1:2 dilution. The calculated TAC was normalized to 1 mg wet weight. Using the protein mask provided in the commercial assay to exclude any protein-based TAC, small molecule TAC was assessed in a subset of five egg samples.

Table 2.1. Specifications of qPCR assays targeting a short (s) and a long (l) segment of the 12S and cytb genes to determine lesion rates per 10 kb, including primer sequences, length of amplicons in base pairs (bp) and and PCR efficiency (Eff). The annealing temperature was 60 °C for all targets.

Target gene (fragment)	Primer	5′-3′ sequence	Size [bp]	Eff [%]
l12S	f	GAACTCAGCAGTGATAGACA	739	86.0
	r	GTACACTTACCATGTTACGA		
s12S	f	GAACTCAGCAGTGATAGACA	249	95.0
	r	CGTAGCTTTCGTGGGTTCAG		
lcytb	f	ACAACGCACTAGTTGACCTA	1066	96.5
	r	GAGAGCCTTGTTTTCAACCCAT		
scytb	f	ATGTTCCATTCTTACCTGA	141	96.4
	r	GAGAGCCTTGTTTTCAACCCAT		

2.5 Data analysis

All data are presented as mean ± standard deviation (SD) of n samples. Statistical analysis was performed with GraphPad Prism (Graph-Pad Software, La Jolla, CA, USA). Data were checked for normality with the Kolmogorov-Smirnov test. For pairwise comparison, nonparametric Mann-Whitney test was used. For multiple comparison, non-parametric Kruskal-Wallis test and Dunn's post-hoc test were carried out. The relation of developmental parameters (fertilization, survival and hatching) and the assessed biomarkers was analyzed using linear regression. Correlation analysis was carried out using nonparametric Spearman correlation.

3 Results

3.1 Egg and embryo development

A constant decline from high fertilization rate (87 ± 19 %; n = 33) to intermediate embryo survival at 24 (86 ± 16 %; n = 31), 48 (79 ± 20 %; n = 30) and 72 h (79 ± 17 %; n = 28) and hatching rate with an average of 75 ± 21 % (n = 30) was observed (Fig. 2.1). This decline was significant (Kruskal-Wallis test: H = 17.8; $P = 0.001$) with embryo survival at 72 h and hatching rate being significantly lower than fertilization rate (Dunn's multiple comparison: $P < 0.05$).

Eggs of three females were post-ovulatory aged ('over-riping') for 4 to 12 h. Only 40 % of these eggs could be fertilized in one female, whereas there was no fertilization observed in the other two. Within 24 h there were no vital embryos left. These eggs are presented separately.

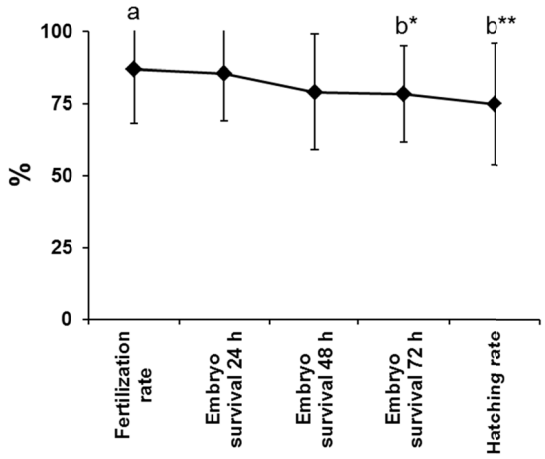


Fig. 2.1. Fertilization (n = 33), survival at 24 h (n = 31), 48 h (n = 30), 72 h (n = 28) and hatching rates (n = 30) (mean ± SD) of pikeperch eggs and embryos as ultimate indicators for egg quality (Kruskal-Wallis test: H = 17.8; $P = 0.001$). Significant differences (Dunn's multiple comparison) are marked by lower case letters and asterisks (*$P < 0.05$; ** $P < 0.01$).

3.2 Fragmentation of mtDNA

UV treatment of eggs caused increasing lesion rates over time in 12S and cytb target regions (Fig. 2.2), but cytb was more sensitive to short-term UV treatment (5, 10 min) than 12S whereas, after 20 min incubation, lesion rate of 12S per 10 kb mtDNA was almost three

times as high (6.03) as lesion rate of cytb (2.33). The qPCR-based quantification was highly reproducible. The average difference between replicates was 0.07 ± 0.06 (s12S), 0.06 ± 0.06 (l12S), 0.08 ± 0.06 (scytb) and 0.12 ± 0.14 (lcytb) (Fig. 2.3).

Average lesion rate per 10 kb mtDNA of 41 observed egg batches was higher in cytb (1.89 ± 2.14) compared to 12S (1.50 ± 1.57), but differences were not significant (Fig. 2.4; Mann-Whitney test: U = 821.5; $P = 0.43$). Lesion rates for both target fragments were highly correlated within egg samples (Spearman correlation: $r = 0.56$; $P < 0.0001$; n = 44; Fig. 2.5). We did not find any significant relationship between mtDNA lesions of 12S and cytb and developmental parameters (Fig. 2.6). Among all developmental parameters, a maximum of 7.54 % of variation in hatching rate could be predicted by lesions in cytb, but the linear regression was not significant (F = 2.28, $P = 0.14$).

The observed mtDNA fragmentation of cytb and 12S within the three batches which underwent post-ovulatory ageing were higher (12S: 2.23 ± 1.13; cytb: 4.02 ± 2.84) compared to other samples. Statistic analysis showed a significant elevation in lesion rates of cytb (Mann-Whitney test: U = 24.0; $P < 0.05$), but not of 12S (Mann-Whitney test: U = 35.5; $P = 0.12$).

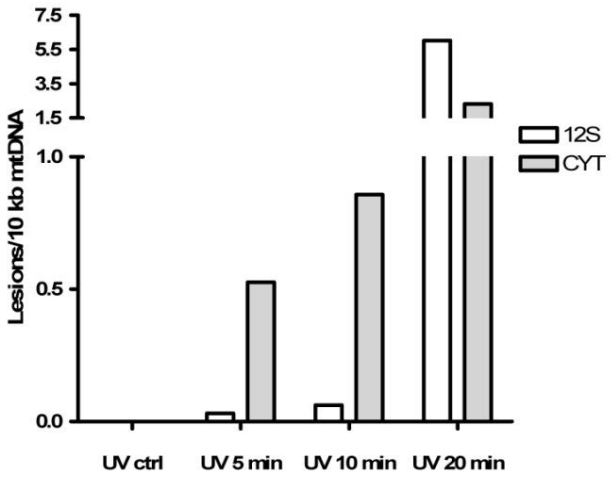


Fig. 2.2. Lesions per 10 kb of 12S and cytochrome b (cytb) of untreated (ctrl) and UV treated egg samples over time. Note the different scaling on the y-axis below and above the gap.

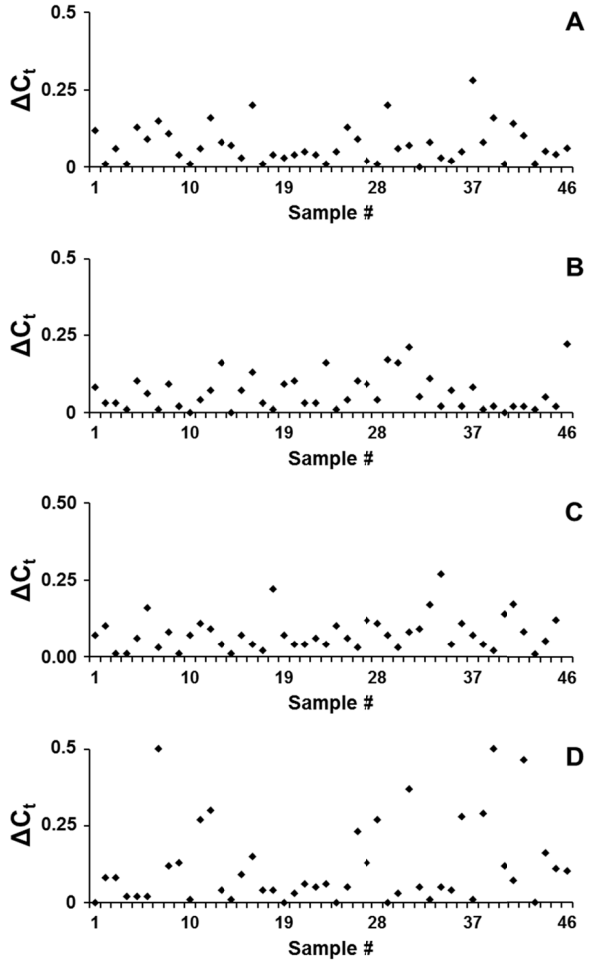


Fig. 2.3. Absolute differences in threshold cycles (ΔC_t) of technical qPCR replicates for all reactions (44 egg samples + 2 calibrators) according to the specific product: 12S short fragment (A), 12S long fragment (B), cytochrome b (cytb) short fragment (C) and cytb long fragment (D).

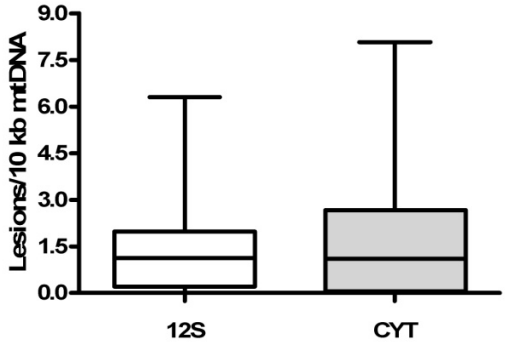


Fig. 2.4. Lesions per 10 kb of 12S and cytochrome b (cytb) for sampled batches of pikeperch eggs (Mann-Whitney test: U = 821.5; *P* = 0.43; n = 41).

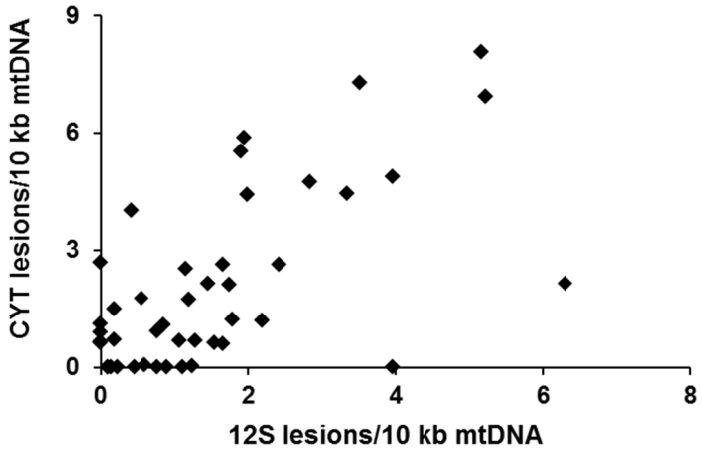


Fig. 2.5. Within-sample correlation of cytochrome b (cytb) and 12S lesions per 10 kb mtDNA for all sampled batches of pikeperch eggs (Spearman correlation: $r = 0.56$; $P < 0.0001$; n = 44).

3.3 Total antioxidant capacity (TAC)

The average TAC was 0.59 ± 0.10 mM trolox equivalent per mg egg wet weight. Values ranged from 0.25 to 0.81 mM trolox equivalent mg^{-1}. The linear regression of TAC versus mtDNA fragmentation was not significant for 12S ($P = 0.79$) and cytb ($P = 0.56$). Linear regression of TAC and developmental parameters likewise did not show significant results and less than 3.5 % variation in could be predicted by TAC. The ratio of small molecules of total antioxidants was relatively stable at 86.36 ± 2.14 %. The TAC (0.51 ± 0.05 mM trolox equivalent mg^{-1}) was significantly lowered in the three aged batches compared to all other batches (Mann-Whitney test: U = 21.5; $P < 0.05$).

4 Discussion

We detected mtDNA lesions within one or both distinct target regions of the mitochondrial genome in all sampled batches of unfertilized pikeperch eggs. The qPCR-based method for the quantification of lesion rates described by Rothfuss et al. (2010) was validated after UV treatment of eggs revealing a substantial increase of lesions upon radiation. The qPCR analysis showed a high precision as confirmed by low deviation of the technical replicates. However, the analysis of the lesion rates of both targets did not explain embryo

developmental success from fertilization to hatching and could thus only predict little variability in egg quality observed here. In contradiction to our hypothesis, elevated egg TAC did not prevent mtDNA fragmentation and ultimately did not improve fertilization, embryo survival or hatching. These results suggest that mtDNA fragmentation, as well as protection via the eggs antioxidant capacity cannot explain the differences in future embryo development until hatching. Hatching was chosen as endpoint to limit the interference of external factors, e.g., feeding, social interactions, which exhibit a large influence on the larvae and herewith possibly mask other effects such as oxidative stress.

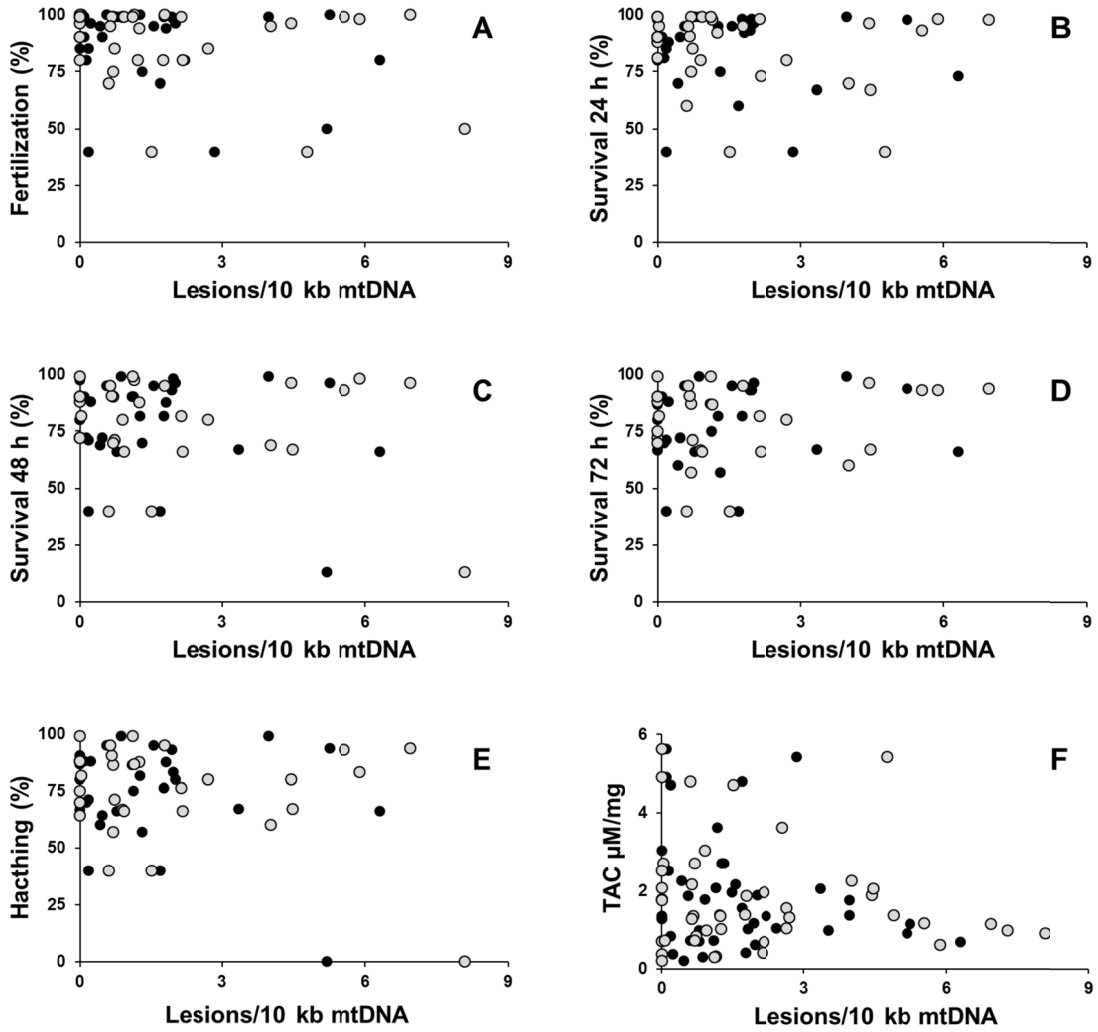


Fig. 2.6. Cytochrome b (cytb; grey circles) and 12S (black circles) lesions per 10 kb mtDNA versus fertilization (A; n = 33), survival at 24 h (B; n = 31), 48 h (C; n = 30), 72 h (D; n = 28), hatching rates (E; n = 30) and versus TAC (F; n = 41) of pikeperch eggs and embryos.

Oxidative stress can potentially cause detrimental damage to the inherent genomic information of an oocyte and can consequently affect embryonic development (Dennery, 2007; Guérin et al., 2001; Wang et al., 2002), potentially inducing apoptosis (Yang et al., 1998). For example, it was shown in *in vitro* cultured bovine embryos that oxygen exposure increased DNA damage resulting in decreased blastocyst formation (Takahashi et al., 2000). However, little is known on effects and consequences of oxidative stress in fish oocytes and embryos, which experience different environmental conditions compared to mammals and other vertebrates.

Mitochondria and hence mtDNA, which are exposed to high levels of reactive oxygen species (ROS) and consequently exhibit higher lesion rates compared to the nuclear genome (Cartón-García et al., 2013; Rothfuss et al., 2010; Yakes and van Houten, 1997). Although, we assessed fragmentation in mtDNA here, lesion rates did only explain little variability of developmental success and are thus not reliable biomarkers for egg quality. It has been reported that different regions of the mitochondrial genome show varying susceptibility towards DNA damage (Rothfuss et al., 2010; Cartón-García et al., 2013). In contrast, several batches of pikeperch eggs observed here showed lesions in one region, but not in the other and there were no significant differences in the average lesion rates between these two regions, which were highly correlated. The UV treatment revealed a time-dependent susceptibility, distinct for both regions. The average degree of the fragmentation in the observed egg batches was low compared to 20 min UV treatment and post-ovulatory aged eggs, which leads to a decline of fertilization success in many fish species as reviewed by Bobe and Labbé (2010) and Migaud et al. (2013). Here, post-ovulatory ageing led to a significant increase of mtDNA lesions of cytb and a lowered TAC. Though not significant, 12S lesion rates were also elevated. Therefore, mtDNA fragmentation and oxidative stress may play an important role during post-ovulatory ageing and subsequently induce embryo mortality. However, the number of aged batches was low and the observed lesion rates in aged eggs did not display the overall highest observed lesion rates, suggesting that oocytes with high lesion rates can still develop into hatched larvae.

This observation on the uncoupling of mtDNA lesions and embryo development can potentially arise from a variety of intrinsic and extrinsic factors. Tentative explanations include the suppression of negative consequences of mtDNA damage by potent inherent repair mechanisms in mitochondria (Rothfuss et al., 2010; Alexeyev et al., 2010).

Furthermore, the large number of mtDNA copies per cell, ~$1.4x10^7$ copies per egg in zebrafish 1 h post fertilization (Artuso et al., 2012), allows for a partial degradation of damaged DNA without the loss of essential genetic information (Alexeyev et al., 2010). This assumption is supported by findings of Artuso et al. (2012) on the decrease of total mtDNA copy numbers during the first 24 h post fertilization in developing zebrafish embryos. Still, it remains unknown whether these degraded copies display the damaged portion of mtDNA.

Pooling of eggs could have potentially masked the observed mtDNA lesion rates of this study. Principally, lesions of mtDNA from individual eggs or even individual mitochondria can cause elevations in the detection of DNA damage in qPCR as performed here. Only single egg analysis could reveal if mtDNA lesions are equally distributed across eggs of one batch and if the observed embryo mortality might be limited to individual eggs with high mtDNA damage resulting in embryo mortality. Within the present study, mtDNA lesions could be detected also in all five egg batches with little embryo mortality (less than 7 % mortality from fertilization to hatching). Therefore, embryos seem to cope with mtDNA damage and hatch successfully.

One could argue that the transport of eggs on liquid nitrogen caused the observed damage of mtDNA as reported (Thomson et al., 2009; Kopeika et al., 2005). However, the effect of freezing on DNA damage remains controversial. While the mitochondrial genome of zebrafish primordial germ cells was generally less sensitive towards freezing compared to nuclear DNA (Riesco and Robles, 2012), Cartón-García et al. (2013) reported significantly higher lesion rates in the mitochondrial genome compared to nuclear markers in cryopreserved gilt-head bream sperm. The latter study also analyzed lesion rates of cytb, which were almost four fold higher compared to the average cytb lesions observed within the present study (Cartón-García et al., 2013), suggesting differing effects in sperm and eggs. Generally, the average lesion rates observed here were low compared to studies, which measured lesion rates in response to cryopreservation (Cartón-García et al., 2013; Riesco and Robles, 2012) and rather match the mtDNA fragmentation observed by Rothfuss et al. (2010) in response to 100 to 500 µM H_2O_2 treatment. Such correspondence suggests substantial damage within the eggs observed within the present study, but it is not clear whether this damage was caused by storage in liquid nitrogen or oxidative stress prior to freezing.

Pérez-Cerezales and colleagues (2009) studied DNA damage in cryopreserved sperm of rainbow trout *Oncorhynchus mykiss* and suggested that other mechanisms apart from

oxidative stress, such as mechanical injuries, may be involved in DNA damage during cryopreservation. This could explain for the observed uncoupling of TAC and mtDNA lesions. However, a considerable number of eggs showed no lesions of either one or only minor lesions of both observed target regions here. Therefore, it is unlikely that transport in liquid nitrogen alone led to a general increase in lesion rates.

It is noticeable that the observed fragmentation within the mitochondrial genome, as well as the developmental success of embryos could not be explained by the TAC of the eggs. We have hypothesized that high levels of inherent TAC can potentially reduce damage caused by oxidative stress and are thus beneficial for embryo survival, but no such relation could be observed. In birds, a correlation of the antioxidant capacity of the eggs and the oxidative status of the females has been reported (Costantini et al., 2010). Further, nutrition is presumably one of the most important factors modulating TAC, but others, such as age and health status will also modulate TAC and contribute to intraspecific variability (Costantini et al., 2010; Ebeid, 2011). Here, a relatively strong variation in egg TAC between females was observed. Since broodstock fish stripped within the present study did not experience differing rearing or dietary conditions, the measured high inter-individual variability of TAC is surprising. Here, endogenous synthesis of proteins with antioxidant effects, differential utilization of antioxidants such as unsaturated fatty acids or vitamins potentially contributed to the individual variability. Generally, a positive effect of dietary antioxidants on embryo development in fish can be assumed (Izquierdo et al., 2001; Palace and Werner, 2006) and several studies demonstrated the relevance of oxidative stress in early embryogenesis (cf. above). TAC offers protection from oxidative stress and previous studies demonstrated a relation of nutrition and TAC (Cao et al., 1998; Ebeid, 2011; Kusano and Ferrari, 2008). However, it remains to be demonstrated that TAC provides effective protection against oxidative stress under hatchery conditions, or predominantly affects other mechanisms during embryo development, such as redox-regulated transcription factors (Dennery, 2007). In addition, it is possible that the observed variation in TAC plays a beneficial role after hatching. The relatively stable ratio of small molecule (non-enzymatic) to protein-derived TAC suggests inter-individual differences in the incorporation of antioxidants to the eggs rather then differences in the endogenous synthesis of specific antioxidants, but without further knowledge on the composition of the measured TAC this remains speculative.

It needs to be determined in future studies, what the magnitude of oxidative stress in fish oocytes is in terms of ROS. In depth analysis of the composition of antioxidants, the determination of repair mechanisms, as well as compensating pathways (e.g., modulation of mitochondria numbers) will help to further understand the consequences for embryogenesis.

Conclusion

The oxidative stress markers assessed here only explained little variability in fertilization, embryo survival and hatching as ultimate gamete quality parameters in pikeperch eggs. This suggests either potent coping mechanisms of embryos compensating for mtDNA lesions, which – in turn – was independent of the antioxidant status or little effect of mtDNA fragmentation during embryogenesis. Therefore, we cannot recommend using TAC or mtDNA lesions as biomarkers to evaluate and predict egg quality in pikeperch.

Acknowledgements

This work was funded by the German Research Foundation (DFG KL 745/6-1). We would like to thank our cooperation partner AquaPri, the members of the IGB working group Applied Aquaculture and Physiology, especially the technical support by E. Kreuz, as well as T. Mehner for his critical discussion while drafting the manuscript.

References

Aitken, R.J., Baker, M.A., 2004. Oxidative stress and male reproductive biology. Reprod. Fert. Develop. 16, 581-588.

Alexeyev, M., Shokolenko, I., Wilson, G., LeDoux, S., 2013. The Maintenance of Mitochondrial DNA Integrity-Critical Analysis and Update. Csh Perspect. Biol. 5.

Artuso, L., Romano, A., Verri, T., Domenichini, A., Argenton, F., Santorelli, F.M., Petruzzella, V., 2012. Mitochondrial DNA metabolism in early development of zebrafish (*Danio rerio*). Bba-Bioenergetics 1817, 1002-1011.

Bobe, J., Labbe, C., 2010. Egg and sperm quality in fish. Gen. Comp. Endocr. 165, 535-548.

Brooks, S., Tyler, C.R., Sumpter, J.P., 1997. Egg quality in fish: what makes a good egg? Rev. Fish. Biol. Fisher. 7, 387-416.

Cao, G.H., Booth, S.L., Sadowski, J.A., Prior, R.L., 1998. Increases in human plasma antioxidant capacity after consumption of controlled diets high in fruit and vegetables. Am. J. Clin. Nutr. 68, 1081-1087.

Carton-Garcia, F., Riesco, M.F., Cabrita, E., Herraez, M.P., Robles, V., 2013. Quantification of lesions in nuclear and mitochondrial genes of *Sparus aurata* cryopreserved sperm. Aquaculture 402, 106-112.

Costantini, D., 2010. Complex trade-offs in the pigeon (*Columba livia*): egg antioxidant capacity and female serum oxidative status in relation to diet quality. J. Comp. Physiol. B 180, 731-739.

Dennery, P.A., 2007. Effects of oxidative stress on embryonic development. Birth Defects Res. C Embryo Today 81, 155-162.

Donaldson, E.M., 1996. Manipulation of reproduction in farmed fish. Anim. Reprod. Sci. 42, 381-392.

Ebeid, T.A., 2011. The impact of incorporation of n-3 fatty acids into eggs on ovarian follicular development, immune response, antioxidative status and tibial bone characteristics in aged laying hens. Animal 5, 1554-1562.

FAO, 2014. The State of World Fisheries and Aquaculture. Food and Agriculture Organization of the United Nations. Rome, 2014.

Ghiselli, A., Serafini, M., Natella, F., Scaccini, C., 2000. Total antioxidant capacity as a tool to assess redox status: critical view and experimental data. Free Radical Biology and Medicine 29, 1106-1114.

Guerin, P., El Mouatassim, S., Menezo, Y., 2001. Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. Hum. Reprod. Update 7, 175-189.

Gurler, H., Calisici, O., Bollwein, H., 2015. Inter- and intra-individual variability of total antioxidant capacity of bovine seminal plasma and relationships with sperm quality before and after cryopreservation. Anim. Reprod. Sci. 155, 99-105.

Izquierdo, M.S., Fernandez-Palacios, H., Tacon, A.G.J., 2001. Effect of broodstock nutrition on reproductive performance of fish. Aquaculture 197, 25-42.

Kopeika, J., Zhang, T.T., Rawson, D.M., Elgar, G., 2005. Effect of cryopreservation on mitochondrial DNA of zebrafish (*Danio rerio*) blastomere cells. Mutat. Res-Fund. Mol. M. 570, 49-61.

Kusano, C., Ferrari, B., 2008. Total antioxidant capacity: a biomarker in biomedical and nutritional studies. J. Cell Mol. Biol. 7, 1-15.

Mahfouz, R., Sharma, R., Sharma, D., Sabanegh, E., Agarwal, A., 2009. Diagnostic value of the total antioxidant capacity (TAC) in human seminal plasma. Fertil. Steril. 91, 805-811.

Metcalfe, N.B., Alonso-Alvarez, C., 2010. Oxidative stress as a life-history constraint: the role of reactive oxygen species in shaping phenotypes from conception to death. Funct. Ecol. 24, 984-996.

Migaud, H., Bell, G., Cabrita, E., McAndrew, B., Davie, A., Bobe, J., Herraez, M.P., Carrillo, M., 2013. Gamete quality and broodstock management in temperate fish. Rev. Aquacult. 5, S194-S223.

Mylonas, C.C., Fostier, A., Zanuy, S., 2010. Broodstock management and hormonal manipulations of fish reproduction. Gen. Comp. Endocr. 165, 516-534.

Palace, V.P., Werner, J., 2006. Vitamins A and E in the maternal diet influence egg quality and early life stage development in fish: a review. Sci. Mar. 70, 41-57.

Perez-Cerezales, S., Martinez-Paramo, S., Cabrita, E., Martinez-Pastor, F., de Paz, P., Herraez, M.P., 2009. Evaluation of oxidative DNA damage promoted by storage in sperm from sex-reversed rainbow trout. Theriogenology 71, 605-613.

Riesco, M.F., Robles, V., 2012. Quantification of DNA damage by q-PCR in cryopreserved zebrafish Primordial Germ Cells. J. Appl. Ichthyol. 28, 925-929.

Rothfuss, O., Gasser, T., Patenge, N., 2010. Analysis of differential DNA damage in the mitochondrial genome employing a semi-long run real-time PCR approach. Nucleic Acids Res. 38.

Sawyer, D.E., Mercer, B.G., Wiklendt, A.M., Aitken, R.J., 2003. Quantitative analysis of gene-specific DNA damage in human spermatozoa. Mutat. Res-Fund. Mol. M. 529, 21-34.

Schwartz, M., Vissing, J., 2002. Paternal inheritance of mitochondrial DNA. New Engl. J. Med. 347, 576-580.

Takahashi, M., Keicho, K., Takahashi, H., Ogawa, H., Schultz, R.M., Okano, A., 2000. Effect of oxidative stress on development and DNA damage in in-vitro cultured bovine embryos by comet assay. Theriogenology 54, 137-145.

Thomson, L., Fleming, S., Aitken, R., De Iuliis, G., Zieschang, J.-A., Clark, A., 2009. Cryopreservation-induced human sperm DNA damage is predominantly mediated by oxidative stress rather than apoptosis. Hum. Reprod. 24, 2061-2070.

Tice, R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J., Sasaki, Y., 2000. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ. Mol. Mutagen. 35, 206-221.

Wang, X., Falcone, T., Attaran, M.D., Goldberg, J.M., Agarwal, A., Sharma, R.K., 2002. Vitamin C and Vitamin E supplementation reduce oxidative stress-induced embryo toxicity and improve the blastocyst development rate. Fertil. Steril. 78, 1272-1277.

Wolff, J.N., Gemmell, N.J., 2008. Lost in the zygote: the dilution of paternal mtDNA upon fertilization. Heredity 101, 429-434.

Yakes, F.M., VanHouten, B., 1997. Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress. P. Natl. Acad. Sci. USA 94, 514-519.

Yang, H.W., Hwang, K.J., Kwon, H.C., Kim, H.S., Choi, K.W., Oh, K.S., 1998. Detection of reactive oxygen species (ROS) and apoptosis in human fragmented embryos. Hum. Reprod. 13, 998-1002.

Chapter III

Synthesis: Interactions of oxidative stress, maternal characteristics and biochemical egg composition in pikeperch

Synthesis: Interactions of oxidative stress, maternal characteristics and biochemical egg composition in pikeperch

Chapter I and II of this thesis are research papers dealing with egg quality in pikeperch. It was shown in chapter I that maternal characteristics are major determinants of egg quality. Furthermore, it could be observed that several aspects of oocyte composition are closely correlated, such as cortisol levels and FA profiles. Within the following chapter, a synthesis is provided analyzing and discussing the interactions of oxidative stress, assessed as lesion rates in mtDNA, as well as antioxidant capacity (chapter II), maternal characteristics and egg quality parameters as presented in chapter I. Additional results are presented considering the respective methodology.

1 Oxidative stress and maternal characteristics

Within chapter I, egg developmental potential, oocyte composition and diameter were analyzed against maternal traits, as well as effects of out-of-season spawning and spawning time. It was shown that maternal traits, especially female length, exert significant adverse effects on egg development until hatching, whereas out-of-season spawning had only a negligible impact. Strip-spawning history of females resulted in increased variability of egg quality, but these effects could rather be explained by female length. Additionally, the spawning time into the season was negatively correlated egg developmental success (fertilization, embryo survival at 24 h), but high quality gametes were still obtained towards late season. Furthermore, maternal traits and spawning time affected the biochemical composition of the oocytes (mRNA, cortisol and specific polar FA content).

The second chapter dealt with potential biomarkers of oxidative stress. In the respective publication, these results were not linked to maternal and broodstock characteristics. Therefore, the data regarding mtDNA fragmentation and TAC presented in chapter II were analyzed against the broodstock characteristics (spawning time, history and seasons, female length) using the methods presented in chapter I. Measurements of egg batches, which

underwent post-ovulatory ageing were excluded from the analysis since they showed significantly higher mtDNA lesions and lower TAC.

There were no significant differences in TAC (Student's t-test: $p > 0.05$) or mtDNA lesion rates of 12S and cytb (Mann-Whitney test: $p > 0.05$) between females stripped for the first time and successively stripped females. Season analysis showed no difference in markers of oxidative stress and antioxidants across the six observed spawning seasons (Kruskal-Wallis test: $p > 0.05$) or significant correlations with female length or spawning time (TAC, Pearson's correlation: $p > 0.05$; mtDNA lesions, Spearman's correlation: $p > 0.05$). Interestingly, there was a positive correlation between cytb lesions and fecundity (Spearman's correlation: $\rho = 0.32$; $p < 0.05$), whereas 12S (Spearman's correlation: $p > 0.05$) and TAC (Pearson's correlation: $p > 0.05$) did not show this. While fecundity was positively correlated with hatching rate whereas indicators of egg developmental potential were independent of mtDNA lesions, the underlying physiological mechanisms affecting egg development remain hidden by the mere observation of these two parameters. Here, the relation of markers of oxidative stress and other biochemical egg components deliver – to a certain extent – further insights.

2 Oxidative stress and egg composition

The interactions of markers of oxidative stress and oocyte antioxidant status and other parameters presented in chapter I were analyzed according to the respective methodology. Significant correlations between TAC, as well as lesion rates of cytb and – to a lesser extent – 12S mtDNA and other egg parameters (dry weight and protein content, absolute and relative content of specific FA) could be observed. The results of these inter-correlations between egg parameters are listed in table 3.1. Interestingly, there was a strong divergence in the occurrence of correlations with FA content between lesion rates of the two observed regions of mtDNA (12S and cytb), which in turn were highly correlated.

The interactions of dietary FA, especially HUFA, with oxidative stress and mitochondria activity have been studied in several fish species. It was shown in salmon (Østbye et al., 2011), rainbow trout (Almaida-Pagán et al., 2015; Bellagamba et al., 2011) and zebrafish (Betancor et al., 2015) that FA levels, especially n-3 HUFA, affect mitochondrial lipid composition. Kjær et al. (2008) found that high levels of n-3 HUFA in the liver were

associated with increased oxidative stress assessed via superoxide dismutase activity in salmon. In addition, high n-3 HUFA levels exerted adverse effects on hepatic mitochondria functions, such as FA β-oxidation (Kjær et al., 2008). Almaida-Pagán et al. (2015) observed changes in mitochondrial gene expression in response to different diets and suggested long-term effects on electron transportation. Betancor and colleagues (2015) detected higher lipid peroxidation in zebrafish mitochondria caused by a DHA enriched rapeseed oil diet. They concluded that increased incorporation of DHA, which is prone to peroxidation, into the mitochondrial membrane inflicts higher levels of oxidative stress leading to macromolecular (incl. DNA) damage (Betancor et al., 2015). This assumption is supported by Bellagamba et al. (2011), who suggested a critical role of lipids in oxidatively induced DNA damage in rainbow trout mitochondria. Consequently, it was recommended that high dietary HUFA levels should be accompanied by high dietary levels of antioxidants to cope with such adverse effects (Kjær et al., 2008; Sargent et al., 2002; Stephan et al., 1995).

It can be assumed that the results here were caused by similar interactions. Oocytes rich in n-3 HUFA showed higher lesion rates in the cytb region of the mtDNA. However, several aspects of the observed relation of mtDNA damage and FA profiles appear puzzling. In addition, no relation of TAC and mtDNA damage could be detected despite variation in the antioxidant levels. Therefore, the following mechanisms may have additionally contributed to these observations.

Not only polar HUFA, but also polar SFA and neutral MUFA and HUFA were positively correlated with cytb, but not with 12S lesion rates. Possibly, oxidative stress associated with FA peroxidation is predominantly affecting the cytb region of the mtDNA. As discussed in chapter II, different regions of the mtDNA show differing susceptibility towards oxidative stress. Furthermore, different mtDNA regions may be more prone towards differing stressors, such as UV light as applied here or H_2O_2 as applied by Rotfhuss et al. (2010).

It could be argued that fragmentation of the cytb, but not of the 12S region, led to a perturbation of mitochondrial functioning consequently affecting FA composition. Parallel to n-3 HUFA induced damage mitochondrial activity may have caused increased levels of reactive oxygen species additionally causing lesions in the mtDNA. As a result, FA accumulated in the oocyte. This could explain for the positive relation of mtDNA lesions and absolute contents of SFA, which are preferred targets for energy provisioning compared to HUFA (Tocher, 2003). Alternatively, it may be suggested that mtDNA fragmentation is

caused by the involvement of mitochondria in FA desaturation and elongation (Cook and McMaster, 2002). However, this assumption appears unlikely since it was shown in zebrafish that such FA modulation does not occur in embryos until ~12 h post-fertilization (Monroig et al., 2000).

Table 3.1. Results of significant inter-correlations of egg parameters as presented in chapter I and II. The coefficient of significant correlations is indicated (Spearman or Pearson, the latter in brackets). The level of significance is indicated by asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Parameter		Lesions per 10 kb 12S	Lesions per 10 kb cytb	TAC
DW content (%)				0.57***
Protein content (%)				0.42*
Polar FA (µg/mg DW)	14:0		0.36*	
	16:0		0.38*	
	18:0		0.32*	
	16:1(n-7)			0.41**
	18:1(n-9)			(0.41**)
	20:5(n-3), EPA		0.39**	
	22:6(n-3), DHA		(0.34*)	
	Sum SFA		0.38*	
	Sum MUFA			0.30*
	Sum n-3 HUFA		(0.36*)	
	Total polar FA		0.44**	
Neutral FA (µg/mg DW)	18:1(n-9)		(0.29*)	
	18:2(n-6)		0.38*	
	18:3(n-3)		0.40**	
	18:2(n-6)		0.38*	
	20:1(n-9)	0.37*		
	20:4(n-6), ARA		0.33*	
	20:5(n-3), EPA		0.38*	
	22:1(n-9)			(-0.29*)
	Sum MUFA		0.29*	
	DHA/EPA		-0.32*	

Abbreviations: cytb, cytochrome b; TAC, total antioxidant capacity; DW, dry weight; FA, fatty acid; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; HUFA, highly unsaturated fatty acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; ARA, arachidonic acid.

By analysis of the present dataset, it remains unknown whether mitochondrial dysfunction caused by mtDNA damage resulted in changes in the FA profiles or if differential incorporation of FA led to higher levels of oxidative stress subsequently causing mtDNA lesions (Fig. 3.1). There are legit arguments supporting either mechanism. Potentially, the observations are a result of a combination of these processes, further magnifying changes in FA profiles.

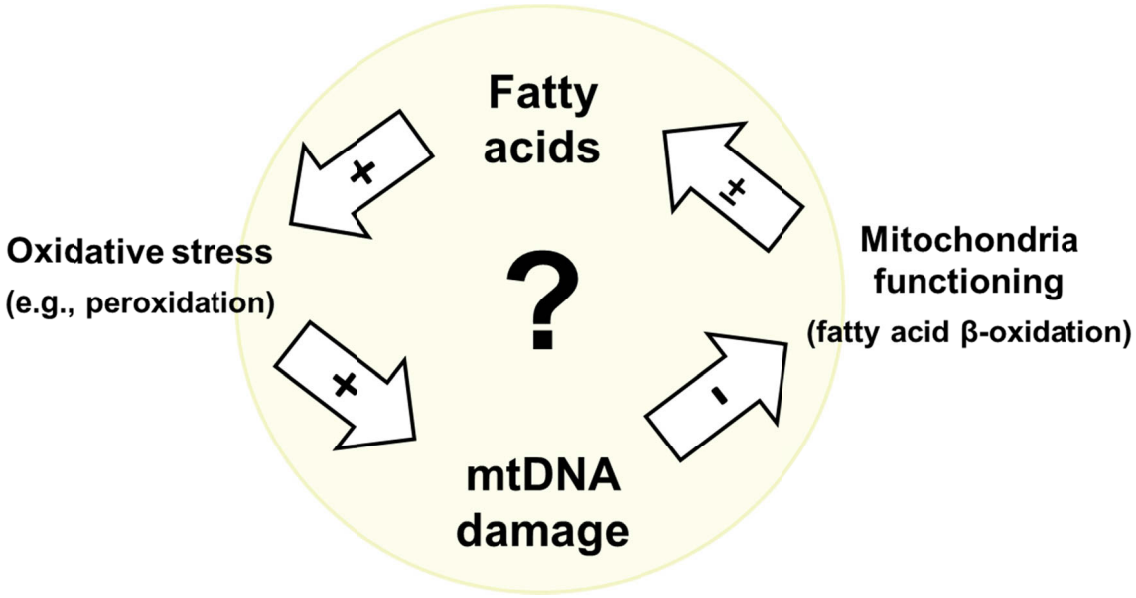


Fig. 3.1. Potential interactions between fatty acid (FA) profiles and mtDNA damage in pikeperch oocytes. Mitochondria dysfunction caused by mtDNA damage potentially alters the FA composition and/or higher levels of FA, especially highly unsaturated FA, are causing increased levels of oxidative stress resulting in higher lesion rates of specific mtDNA regions.

Still, these potential interactions do not explain for the correlations between cytb lesion rates and neutral FA. As discussed in chapter I, neutral FA are predominantly stored in the oil globule and not utilized until hatching. An involvement of neutral FA in mitochondrial integrity or functioning in the oocyte can therefore largely be excluded. However, another explanatory mechanism could be suggested involving maternal coping strategies (cf., the general discussion).

Regarding the antioxidant status of the oocytes, it was shown in chapter II that the majority of the TAC can be attributed to small molecules and not to proteins. The main non-enzymatic antioxidant in fish eggs is vitamins E, but to a certain extent also vitamins A and C (ascorbic acid), as well as carotenoids (Dabrowski and Ciereszko, 2001; Palace and Werner,

2006). Still, given the strong correlation of protein and DW content, TAC levels significantly increased with both egg parameters. Such a positive relation of protein content and TAC was previously observed in sperm of Eurasian perch (Slowińska et al., 2013). Similarly, the majority of TAC derived from small molecules (Slowińska et al., 2013). Due to their vulnerability towards peroxidation through reactive oxygen species, it was expected that HUFA levels would be higher in oocytes with increased levels of antioxidants (Sargent et al., 2002). However, no such relation could be observed here. In contrast, polar MUFA were associated with elevated TAC, while the neutral MUFA 22:1(n-9) was negatively correlated with antioxidant status. It remains unclear, whether these polar MUFA directly act as antioxidants. Here, elevated polar 18:1(n-9) indicate degradation of neutral 22:1(n-9). However, without further knowledge of specific storage location and utilization of the neutral 22:1(n-9) it remains unknown if the degradation of longer chain MUFA is contributing to the TAC resulting in increased accumulation of shorter chain MUFA.

Interestingly, there were no parallel correlations between any specific FA with mtDNA fragmentation and TAC. This becomes particularly obvious in regard to polar FA where TAC was correlated with MUFA, whereas lesions in cytb were correlated with SFA and HUFA. This pattern suggests complex interactions in FA metabolism during oxidative stress contributing to the independence of TAC and mtDNA damage. Therefore, it is likely that mitochondrial markers of oxidative stress as assessed here may possibly not reflect the overall status of oxidative damage in the oocyte. In parallel, total oocyte antioxidants did not prove to be a suitable approach for studying mitochondria based oxidative processes. Further studies are suggested, which target this sensitive relation.

Chapter IV

Management of pikeperch *Sander lucioperca* sperm quality after stripping

Management of pikeperch *Sander lucioperca* sperm quality after stripping

Fabian J. Schaefer, Julia L. Overton, Jiri Bossuyt, Daniel Żarski, Werner Kloas and Sven Wuertz

Journal of Applied Ichthyology (accepted for publication)

Summary

The aim of this study was to identify potential for optimizing the management of sperm quality during commercial reproduction in pikeperch *Sander lucioperca*. Sperm of different males is often pooled prior to fertilization or stored for short periods (hours) until ovulated eggs become available. We applied a novel approach to assess pooling effects by cross-wise transfusion of sperm and seminal fluid (SF) of males with differing initial sperm quality. In addition, we tested the effects of two different buffers (glucose and KCl), as well as the supplementation with melatonin and progesterone (1 mM) to maintain or improve the quality of freshly stripped, as well as incubated (0.5, 1, 2, 4 and 24 h) sperm. Sperm motility and curvilinear velocity (VCL) were measured by computer assisted sperm analysis (CASA). The VCL proved to be a more sensitive, reliable parameter compared to motility, since significant differences occurred up to 3.5 h earlier. Transfusion of SF between low and high quality sperm resulted in a significant decrease in sperm with high initial VCL (7 out of 22 transfusions), whereas VCL of low quality sperm could not be improved. Only in one case, transfusion resulted in an increased VCL. No treatment could prevent a significant quality loss over 24 h or even enhance sperm performance. Conclusively, pooling sperm of males with different quality, as well as short-term storage has a significant negative impact on overall sperm quality. Pooling should only be considered if the sperm quality is known.

1 Introduction

Improvements in recirculating aquaculture technology and rearing protocols have promoted the culture of new temperate fish species, such as pikeperch *Sander lucioperca.* The species is drawing more and more mutual attention of both, research and commercial aquaculture. Most importantly, protocols allowing for multiple out-off-season reproductions have been established (Müller-Belecke and Zienert, 2008; Hermelink et al., 2011), which are successfully applied on a commercial scale. However, controlled reproduction of pikeperch is still suffering from strong variations in gamete and offspring quality, preventing a consistent supply of stocking material for the industry (Żarski et al., 2015). Such variation in reproductive success demonstrates the need for optimization of currently applied protocols.

In hatcheries, it is often critical to time the right moment to strip female fish to avoid a spontaneous release of eggs and uncontrolled fertilization (Żarski et al., 2012). In comparison, it is easier to obtain gametes from males, which are ready to spawn over days prior to the female spawning time. According to current protocols for *in vitro* fertilization in pikeperch hatcheries, either a single male is used or sperm of several males is pooled prior to fertilization aiming at a minimization of fertilization failure caused by individual males with low sperm quality and to enhance genetic diversity. It is currently unknown, which fertilization strategy is advantageous in terms of fertilization success. In addition, sperm often needs to be stored over short periods of time (hours) to ensure availability at time of ovulation. Still, pooling effects on sperm quality remain largely unknown and a decrease in pikeperch sperm quality within hours after stripping has been observed (Křišťan et al., 2014).

The sperm of most teleost fish species is immotile within the testis and is only activated upon contact with water (Cosson, 2004). Subsequently, in most freshwater teleosts, sperm is only motile for a short time compared to other vertebrates, ranging from seconds up to several minutes, dependent on the species as well as external parameters (Billard and Cosson, 1992; Cosson, 2004). The properties of the seminal fluid (SF), such as ion concentrations, pH and osmolality affect sperm motility and subsequently modulate fertilization success (for review Alavi and Cosson, 2005, 2006; Islam and Akhter, 2011). Thus, buffers mimicking the conditions of the SF may allow for a dilution of sperm without activation of spermatozoa and such solutions are already widely used as extenders in cryopreservation (for review Cabrita et al., 2010; Suquet et al., 2000). The incubation of freshly stripped sperm within such

immobilizing buffers allows for a supplementation with substances, which can potentially preserve or even enhance sperm performance and herewith extend the availability of high quality sperm during reproduction.

There is evidence that melatonin can be beneficial to enhance or conserve sperm quality (motility and/or velocity), as observed *in vivo* in killifish *Fundulus heteroclitus* (Lombardo et al., 2014), in ram (Ashrafi et al., 2011) and post-thawed bull semen (Ashrafi et al., 2013), which was associated with a reduction of oxidative stress such as lipid peroxidation, as well as an increase in the total antioxidant capacity and antioxidant enzyme activity. Similarly, melatonin prevents sperm apoptosis in humans after ejaculation (Espino et al., 2010), which is already experimentally applied in assisted reproduction (Bejarano et al., 2014). However, this has been insufficiently documented in fish.

Progestin-mediated stimulation of sperm motility is widely observed in vertebrates and can directly increase sperm motility in fish (Tubbs and Thomas, 2008, 2009; Tubbs et al., 2011). Still, the mechanisms governing this effect are poorly understood, particularly in teleosts. It was shown that short-term (10 min) incubation of human sperm in the presence of progesterone stimulates hypermotility (Uhler et al., 1992) and could be a possible treatment for infertility (Oehninger et al., 1994).

In the present study, we conducted two experiments using stripped pikeperch sperm and assessed sperm quality (motility, velocity) by computer assisted sperm analysis (CASA). In the first experiment, sperm was incubated and repeatedly measured for up to 24 h without further treatment (control), within two buffer solutions (KCl and glucose) derived from species-specific cryopreservation protocols (Bokor et al., 2007), as well as in melatonin and progesterone supplemented KCl buffer. In the second experiment, we applied a novel methodological approach by transfusing sperm and SF. Sperm was incubated (1:10) and repeatedly measured for up to 1 h within the SF of another male with different initial quality (high (> 56 μm s^{-1}) or low (< 28 μm s^{-1}) velocity). These experiments deliver valuable insights to current hatchery practice regarding (I) effects of short-term storage up to 24 h post stripping and (II) pooling of several males prior to fertilization. We hypothesized that (I) the supplementation of buffer solutions and the supplementation with progesterone and melatonin can potentially be beneficial to preserve or even further enhance sperm quality and that (II) the incubation of sperm of one individual with the SF of another individual has the potential to modulate its performance.

2 Materials and methods

2.1 Animals and sperm collection

For sperm exposure experiments, 12 pikeperch males (m1 – m12) were stripped. The males derived from several commercial stocks. Photothermal protocols for reproduction, as well as fish origin and rearing differed with the following similarities. Fish were reared in recirculating aquaculture systems in groups of mixed sexes and maturation was induced after a minimum of three months wintering at temperatures below 14 °C. Afterwards, the water temperature was raised to ~16 °C to initiate spawning. Fish were caught in a net, anaesthetized (according to the local hatchery practice) and the sperm was stripped. Semen was collected using a syringe while applying gentle pressure on the abdomen. The semen was portioned and always kept on ice (4 °C).

2.2 Computer assisted sperm analysis (CASA)

Sperm motility (%) and curvilinear velocity (VCL; μm s^{-1}) were measured with a Sperm Class Analyzer (Microptic) connected to a phase contrast microscope (ECLIPSE Si/Ni; Nikon) with an area scan camera (Ace ACA 780-75gc; Basler). For the analysis, temperature was controlled with a Peltier stage (PE120-XY; Linkam Scientific Instruments) and adjusted to the ambient rearing temperature of the broodstock (16 °C). The tip of a 10 µl pipette was dipped in the semen and immediately transferred to 100 µl of activating solution (distilled water with 1% w/v bovine serum albumin; Sigma-Aldrich). Sperm was activated by gentle mixing with the activating solution for 3 s. Activated sperm was transferred to a disposable four chamber 20 micron Leja slide. CASA measurement was started after the apparent end of the cell drift on the slide, ~10 s after initial activation at 100 X magnification (10x Ph1lense, Nikon). Twenty-five pictures were taken over an entire measurement duration of 1 s. Measurements were performed in duplicate.

A total maximum of 200 spermatozoa was allowed for each individual measurement, but violations of this limit up to ~300 spermatozoa were tolerated after a visual confirmation of the video data (e.g., low overall velocity). Questionable individual measurements were excluded after detailed screening of the video data (e.g., continuous cell drift, high number of spermatozoa, air pockets on the slide). In some cases, this exclusion led to a decrease in

replicate measurements to a minimum of one measurement with a minimum of 94 individual spermatozoa.

2.3 Buffers and sperm quality enhancing supplements

For each of the six spawners (m1 – m6), aliquots of semen were mixed in a ratio of 1:10 with KCl buffer (KCl: 200 mM KCl, 30 mM Tris, pH 8.0), glucose buffer (Glu: 350 mM glucose, 30 mM Tris, pH 8.0), melatonin (Molekula) buffer (Mel: 1 mM melatonin in KCl buffer) and progesterone (Sigma Aldrich) buffer (Prog: 1 mM progesterone in KCl buffer). Thereafter, CASA analysis was carried out immediately after mixing (0 h) and after 0.5, 1, 2, 4 and 24 h, always in the same order. As control, untreated semen of the respective male was analyzed in the same time intervals.

2.4 Seminal fluid transfusion

After initial quality check by CASA, the fish were categorized as either high (VCL > 56 µm s^{-1}) or low quality (VCL < 28 µm s^{-1}), corresponding to a two-fold difference. Approximately 200 - 500 µl (depending on the available volume) of the freshly stripped semen were centrifuged for 5 min at 3,200 g. The clear SF was collected and constantly kept at 4 °C. The SF was checked under the microscope to exclude the presence of spermatozoa. Sperm and SF were incubated (at 4 °C) by mixing untreated sperm in a ratio of 1:10 with SF. Males were stripped successively and transfusion was carried out according to the availability (Table 4.1): (A) six males with high (m7, m9, m10) and low (m8, m11, m12) VCL were used directly after stripping, (B) upon short-term storage (24 h), transfusion was carried out crosswise with aged sperm and SF of three males (m4, m5, m6) with varying initial VCL (one high, two low), (C) with stored sperm (m1; 24 h) and freshly obtained SF (m2), both with high initial VCL. Sperm quality was analyzed by CASA after 5, 15, 30 and 60 min post incubation.

2.5 Data analysis

Replicate measurements were combined for the display of an overall value of percent motile spermatozoa and of mean VCL per male, treatment and incubation time. The VCL data is additionally presented as mean ± standard deviation (SD) of all motile spermatozoa for each measurement. Data was checked for normality by Kolmogorov-Smirnov test. After parametric one-way ANOVA or nonparametric Kruskal-Wallis test, multiple comparisons were performed with Tukey's or Dunn's post-hoc test respectively. Comparisons of two non-parametric data sets were performed with Mann-Whitney test. Statistical analysis was performed with GraphPad Prism (GraphPad Software).

3 Results

3.1 Short-term storage in buffer and sperm quality enhancing supplements

The initial motility in untreated sperm ranged from 30.2 to 97.9% with a mean of 71.9 ± 27.3%. One male (m4) was declared as unfertile and only observed directly after stripping and after 24 h incubation in the respective treatments.

After 4 h and 24 h storage, sperm motility appeared to be different between the control of untreated sperm and the four treatments, but Tukey's multiple comparison test did not indicate significant differences in-between groups (Fig. 4.1). Motility in the control did not decrease significantly from 0 to 24 h (One-way ANOVA: $P = 0.74$). In all other treatments, decrease in motility was significant (One-way ANOVA: $P < 0.05$).

Mean initial VCL was 64.8 ± 39.8 μm s^{-1}. The highest observed individual sperm speed was 167.4 μm s^{-1}. The measured average VCL at 0 h appeared to be lower in Glu compared to other treatments, but the difference was not significant (One-way ANOVA: $P = 0.42$). After only 0.5 h incubation, average VCL was reduced in all treatments ranging from 12.4 μm s^{-1} (Glu) to 40.3 μm s^{-1} (Prog) compared to the control at 68.9 μm s^{-1}, but only the decrease in Glu was significant (Dunn's multiple comparison: $P < 0.05$; Fig. 4.2). After 24 h, all treatments resulted in lower VCL compared to the control (One-way ANOVA: $P < 0.001$). No significant change in VCL over time was observed within the control (Kruskal-Wallis test: $P = 0.47$) and within Glu (Kruskal-Wallis test: $P = 0.20$). For all other treatments, VCL

significantly decreased over time (Tukey's multiple comparison: $P < 0.05$). Still, the time course differed between treatments. A significant decrease, in VCL was first observed in KCl (0.5 h; Tukey's multiple comparison: $P < 0.05$) then in Mel (1 h; Tukey's multiple comparison: $P < 0.05$) and finally in the Prog treatment (4 h; Tukey's multiple comparison: $P < 0.05$).

Analysis of the Prog treatment did not indicate hypermotility upon activation (Mann-Whitney test: $P > 0.05$; n = 6). The initial VCL of Prog treatment was comparable to KCl or the control. After 0.5 h, average VCL was higher in the control in four out of five males (Mann-Whitney test: $P < 0.05$). No significant changes of VCL were detected between Prog and KCl at 0 and 0.5 h of incubation (Mann-Whitney test: $P > 0.05$). In the control, the decrease in VCL from 0 to 24 h was significant in five out of six males (Mann-Whitney test: $P < 0.001$; Fig. 4.3).

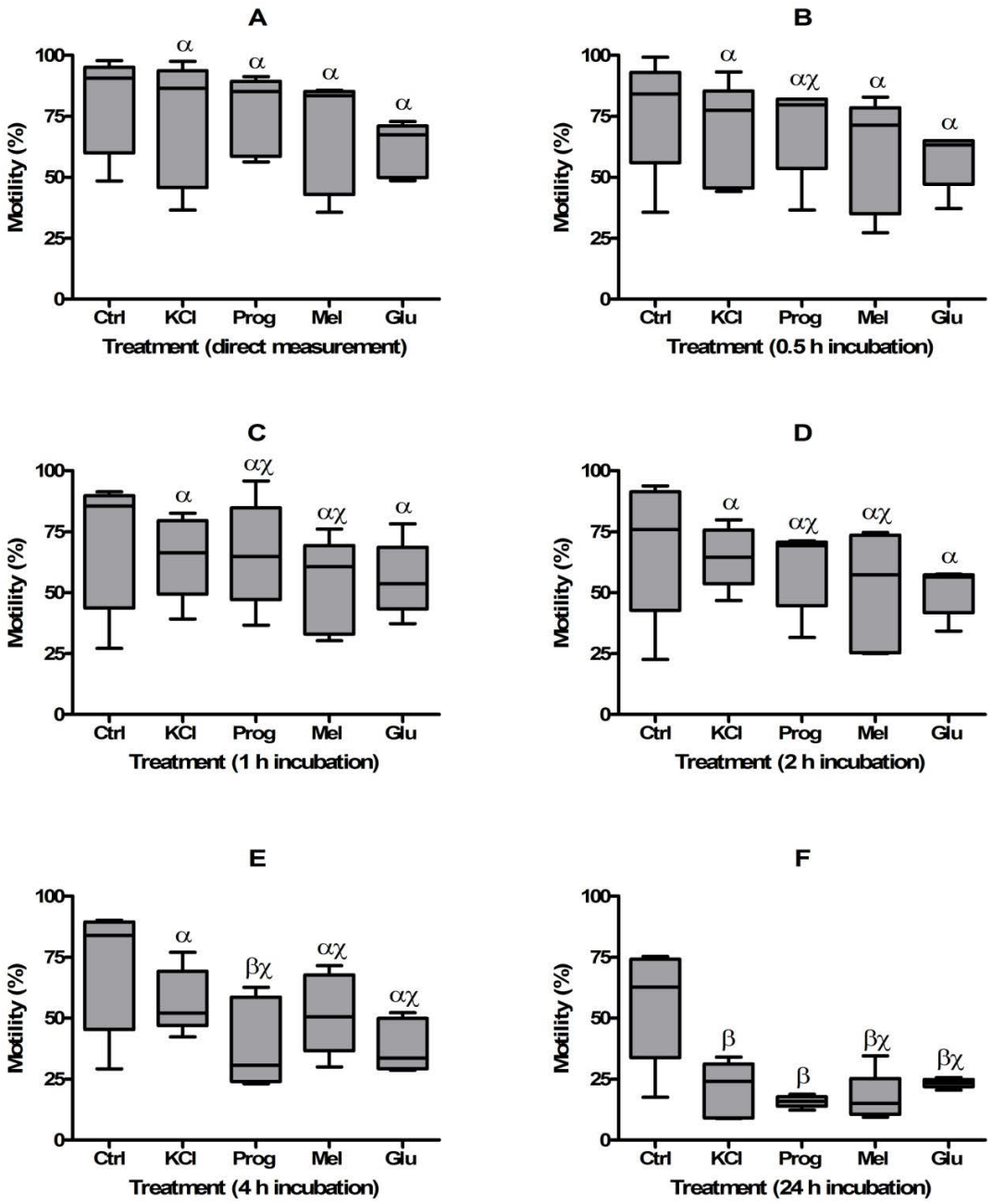


Fig. 4.1. Sperm motility (%) of pikeperch males (n = 6) at different times of incubation (A: 0 h; B: 0.5 h; C: 1 h; D: 2 h; E: 4 h; F: 24 h). Results are grouped per treatment: control of untreated sperm (Ctrl); KCl extender (KCl); 1 mM progesterone in KCl extender (Prog); 1 mM melatonin in KCl extender (Mel); glucose extender (Glu). Significant differences within treatments across time are indicated by greek letters (Tukey's multiple comparison: $P < 0.05$). Columns indicate 25th and 75th percentile and contain the median (line). Whiskers indicate minimum and maximum values.

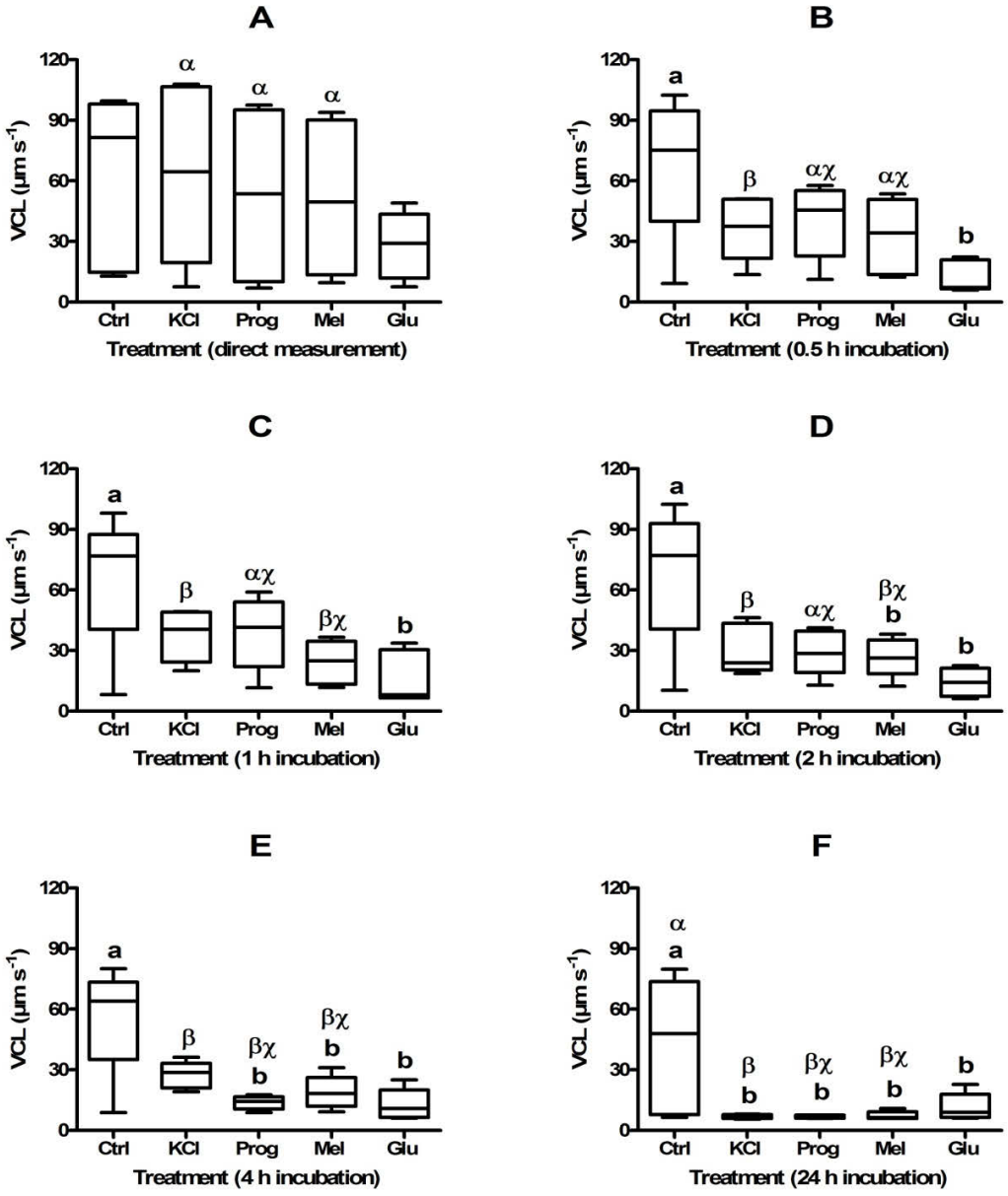


Fig. 4.2. Sperm curvilinear velocity (VCL; µm s^{-1}) of pikeperch males (n = 6) at different times of incubation (A: 0 h; B: 0.5 h; C: 1 h; D: 2 h; E: 4 h; F: 24 h). Results are grouped per treatment: control of untreated sperm (Ctrl); KCl extender (KCl); 1 mM progesterone in KCl extender (Prog); 1 mM melatonin in KCl extender (Mel); glucose extender (Glu). Significant differences between treatments and control are indicated by lower case letters (Dunn's (B, C) or Tukey's (A, D – F) multiple comparison: $P < 0.05$). Significant differences within treatments across time are indicated by greek letters (Tukey's multiple comparison: $P < 0.05$). Columns indicate 25th and 75th percentile and contain the median (line). Whiskers indicate minimum and maximum values.

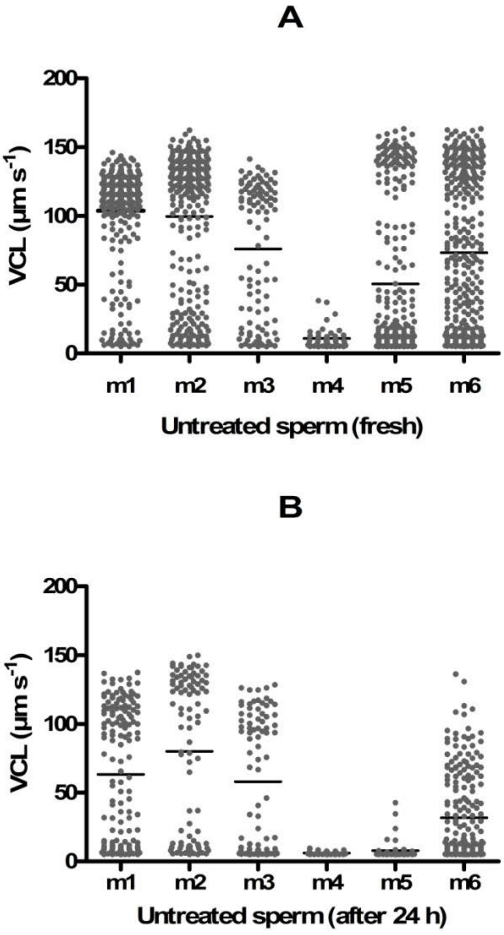


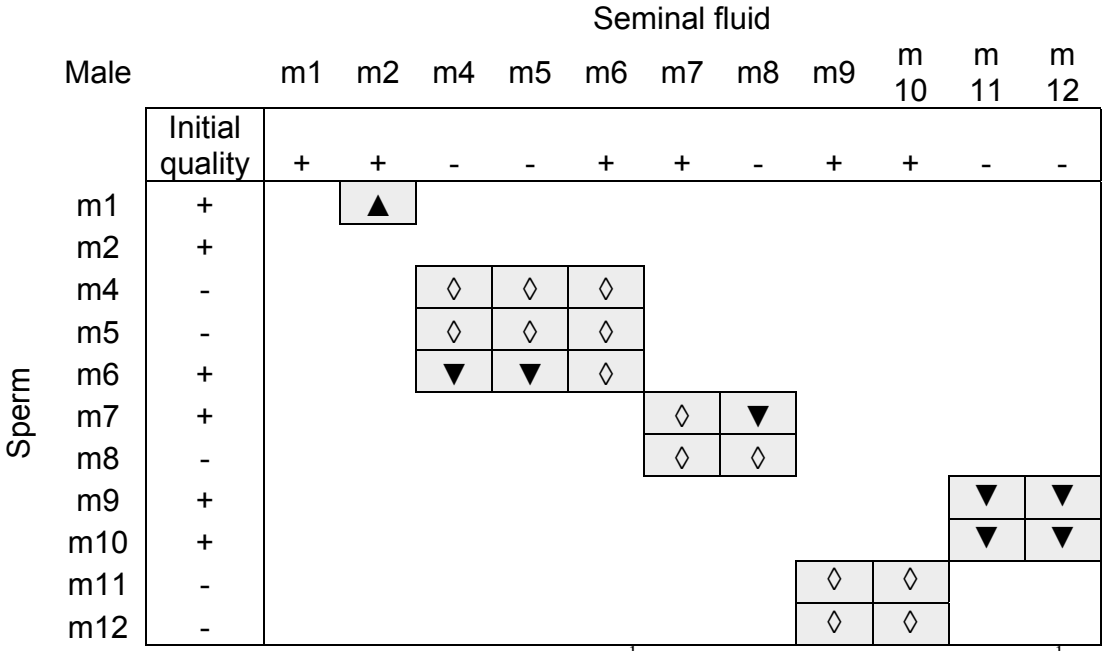
Fig. 4.3. Curvilinear velocity (VCL; µm s^{-1}) of untreated individual spermatozoa of six pikeperch males (m1 – m6) directly after stripping (A) and after 24 h storage (B). Each dot represents a single spermatozoa. Mean VCL is indicated by a line. The VCL significantly decreased during 24 h in all fertile males (m1 – m3, m5, m6; Mann-Whitney test: $P < 0.001$).

3.2 Seminal fluid transfusion

Table 4.1 summarizes 22 transfusions with sperm and SF derived from high (VCL > 56 µm s^{-1}) and low (VCL < 28 µm s^{-1}) quality sperm and the respective effects on initial sperm quality, which were assessed as change in VCL over time (after 5, 15, 30 and 60 min). The incubation of 24 h old sperm with freshly stripped high quality SF resulted in a significant elevation of VCL after 15 min incubation (Dunn's multiple comparison: $P < 0.001$; Fig. 4.4). In six cases (high VCL sperm versus low VCL SF), transfusion revealed a decrease in sperm VCL already after 5 min incubation (Dunn's multiple comparison: $P < 0.001$). The

transfusion of sperm of m10 (high quality) and SF of m12 (low quality) resulted in a delayed significant reduction of VCL after 15 min (Dunn's multiple comparison: $P < 0.001$). Fourteen transfusions did not reveal a change in VCL. Intra-individual (sperm and SF derived from the same individual) transfusion (n = 5) did not result in a change of VCL, assessed twice in freshly stripped sperm and three times after 24 h storage. The SF of high quality males had no positive effect on the VCL of low quality sperm (n = 7).

Table 4.1. Summary of effects of seminal fluid transfusion experiments on sperm curvilinear velocity (VCL). Initial sperm VCL was classified as high (+) or low (-) quality* at beginning of the experiment. Respective transfusions between sperm and seminal fluid (1:10) are indicated by grey boxes. Effects of transfusion on VCL are indicated as positive (▲), negative (▼) or with no significant effect (◊).



*High quality (+): VCL > 55 µm s^{-1}; low quality (-): VCL < 28 µm s^{-1}

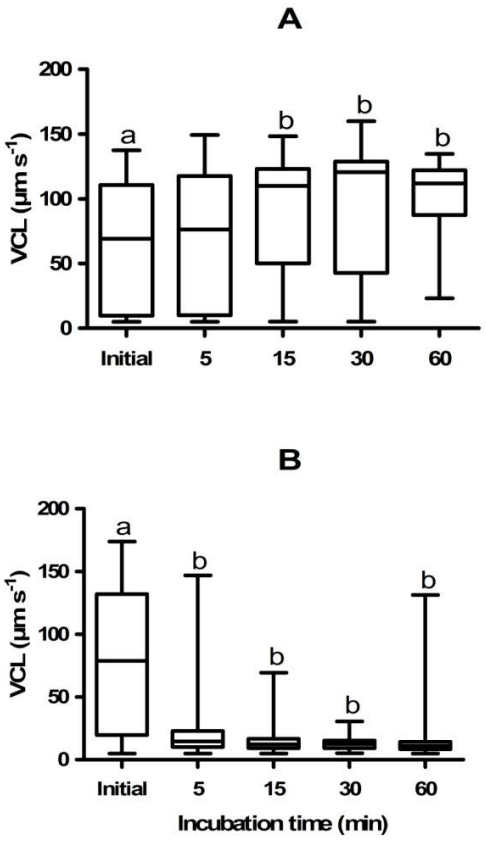


Fig. 4.4. Sperm curvilinear velocity (VCL; µm s^{-1}) of pikeperch sperm prior to (initial) and after seminal fluid (SF) transfusion (1:10 incubation of sperm in SF) repeatedly measured after 5, 15, 30 and 60 min incubation time (cf., table 4.1). A: Untreated sperm (m1; 24 h old control; high initial quality) incubated in the SF of m2 (fresh; high quality). B: Example of decline in VCL of untreated sperm (m9; high quality) in SF of m11 (low quality). Significant differences in VCL compared to initial VCL are indicated by lower case letters (Dunn's multiple comparison: $P < 0.001$). Columns indicate 25th and 75th percentile and contain the median (line). Whiskers indicate minimum and maximum values.

4 Discussion

In pikeperch, low sperm motility was reported, limiting the propagation of this species in aquaculture (Bokor et al., 2007, 2008; Cejko et al., 2008). Here, we aimed at an optimization of hatchery protocols regarding short-term storage, as well as sperm pooling effects. Neither inactivation buffer nor the supplementation with melatonin or progesterone prevented a loss of sperm quality or enhanced sperm performance over the period of 24 h after stripping. In contrast, overall quality (motility and VCL) of untreated sperm (control) did not decrease

significantly over time. However, individual analysis showed a significant drop in VCL after one day in all fertile males. As a consequence, sperm should be used within 24 h for fertilization in pikeperch. The transfusion experiments clearly demonstrated the important role of the SF with regard to sperm performance. Interestingly, it was possible to restore sperm quality (VCL) after 24 h storage in one male by using fresh, high quality SF. However, crosswise transfusion showed that SF of high quality sperm does not enhance low sperm quality. In contrast, the incubation of good performing sperm in low quality SF led to a significant decrease in VCL. Overall, the experiments conducted here deliver valuable insights for hatchery practice, particularly for breeding programs where proper distribution of genetic material is paramount.

Significant decline in VCL became visible already after 0.5 h incubation, but motility remained on comparable levels until 24 h. Quantification of VCL is based on single spermatozoa and thus represents a mean of several hundred sperm cells, which provides more reproducible, differentiated data compared to the ratio of moving and not moving cells. Here, analysis of VCL proved to be a more time-sensitive quality parameter compared to motility. Congruently, VCL was identified as one of the most useful parameters for the determination of sperm quality in fish as reviewed by Rurangwa et al. (2004). It was shown in a variety of fish species, such as catfish *Clarias gariepinus* (Rurangwa et al., 2001), carp *Cyprinus carpio* (Linhart et al., 2000), rainbow trout *Oncorhynchus mykiss* (Lahnsteiner et al., 1998), cod *Gadus morhua* (Rudolfsen et al., 2008), Atlantic salmon *Salmo salar* (Gage et al., 2004) and walleye *Sander vitreus*, the close North American relative to pikeperch (Casselman et al., 2006), that sperm velocity is positively correlated with fertilization and/or hatching rate. Therefore, a positive relation of the VCL observed here and the fertilization abilities of the respective males can be assumed.

Differences in methodologies complicate a direct comparison with previous efforts, which aimed for evaluating buffers for short-term storage of diluted pikeperch sperm (Korbuly et al., 2009). Korbuly and colleagues (2009) observed a drop of 20 to 35% in sperm motility from 1 to 3 h incubation in three dilution buffers used (human normal saline; fish physiological saline; Tris 2.42 g L^{-1}, glycine 3.75 g L^{-1}, NaCl 5.52 g L^{-1}, KCl 2.0 g L^{-1}), but not in Ringer's solution and phosphate buffered saline (PBS). Generally, a direct comparison of sperm parameters is difficult, due to the effects of temperature (during sperm activation) as reviewed by Alavi and Cosson (2005). Still, the values observed here are in the range of

previous reports on pikeperch motility and VCL. Blecha et al. (2015) reported average sperm motility ranging from 69 to 95% with an average VCL of 159 to 219 µm s^{-1}. Sarosiek et al. (2016) observed motility ranging from ~35 to 75% and an average VCL of ~75 to 125 µm s^{-1}. Křišťan and colleagues conducted a study on the effects of activation media at different storage times (up to 48 h at 6 °C) and reported sperm motility rates of 92 to 99% and velocities of 161 to 204 µm s^{-1} directly after stripping (Křišťan et al., 2014). Motility dropped stepwise over 6, 24 to 48 h with 6 to 30% of motile sperm and a velocity of 9 to 13 µm s^{-1} after 48 h (Křišťan et al., 2014). These patterns are similar to our study indicating a drastic loss of sperm quality within a few hours after stripping, but again, direct comparison – especially of VCL – is questionable due to the different methodologies applied, as well as differences in fish origin and rearing. Cejko et al. (2008) reported differences in sperm motility (no VCL data available) in-between differently reared males (ponds, net cages, recirculating system). Here, fish were sampled from several stocks and rearing conditions, as well as reproduction protocols differed slightly. Due to the strong inter-individual variability in VCL, we suggest that male-specific characteristics (e.g., size, age, condition) rather than applied rearing protocols determine sperm traits. However, this cautious suggestion requires further research. Here, inter-individual transfusion of sperm and SF was always carried out with males kept under the same rearing conditions.

In contrast to our hypothesis, it was not possible to counteract a loss of sperm quality during short-term storage using inactivation buffers (glucose or KCl) or by supplementation with enhancers (melatonin, progesterone). Still, activation of sperm was possible at all times in all treatments, suggesting that both buffers did not induce sperm activation prior to measurement. Despite all efforts have been made to avoid urine contamination, it is still possible that urine caused the observed inter-individual differences as reviewed by Rurangwa et al. (2004). In contrast to glucose buffer, KCl buffer provided better motility and VCL, which were comparable to untreated sperm immediately after mixing. Here, neither treatment improved sperm quality, on the contrary, motility and VCL even decreased compared to untreated sperm.

The supplementation of progesterone and melatonin as motility enhancers did not result in hypermotility, higher ratios of motile sperm or prevented quality loss over time. In human sperm, progesterone induces an increase in velocity (hypermotility) through the activation of Ca^{2+} channels (Calogero et al., 2000; Lishko et al., 2011). In male fish, progestins mediate the

final maturation (Scott et al., 2010) and enhancing effects on sperm traits have also been reported. In spotted seatrout *Cynoscion nebulosus* 20nM 20β-S induced an increase in sperm motility after 1 min incubation via the membrane progestin receptor (Tubbs and Thomas, 2008), similar to the observations in Atlantic croaker *Micropogonias undulatus* (Tubbs and Thomas, 2009). These receptors were also identified in zebrafish *Danio rerio* and pufferfish *Fugu rubripes* (Zhu et al., 2003; Hanna et al., 2006). Progestin receptors shows binding affinities for their natural progestin hormones including progesterone (Hanna et al., 2006; Thomas et al., 2007). However, no receptor binding studies have been carried out in pikeperch and the natural progestin in males of this species has not been identified. Assuming cross-reactivity, we used progesterone, but did not observe an effect with the present study. Here, the only detectable effect of progesterone treatment was a delay in the decrease of VCL until 4 h after stripping, compared to a decrease after 0.5 h in pure KCl buffer. In line with our observations, Murack et al. (2011) did not detect any change in sperm performance upon short-term (minutes) *in vitro* exposure to progesterone (1, 10, 100 nM) in fathead minnow *Pimephales promelas* sperm. They suggested that either there is no receptor capable of binding progesterone in this species, progesterone as precursor is not converted into more active steroids (e.g., 17α,20β-P or 20β-S) or the receptors act not fast enough to quickly mediate changes in the velocity (Murack et al., 2011). The latter suggestion seems unlikely, since we observed effects of progesterone treatment for up to 24 h. It is however possible that the observed delay in the decrease of VCL upon progesterone treatment is due to slow conversion into more active steroids, which – in turn – is masked by storage effects. A broader range of concentrations, as well as of progestins, including 17α,20β-P or 20β-S, should be assessed to clarify, if induction of hypermotility by progestins is present in pikeperch.

Melatonin is an important regulator of the endocrine system in fish including the regulation of reproduction, interlinking photoperiod and the timing of endogenous physiological processes, such as gonad maturation and spawning (review by Falcon et al., 2010). Lombardo and colleagues (2014) exposed killifish to melatonin-supplemented rearing water and observed a greater portion of motile sperm with significantly elevated VCL. They suggested that the melatonin acts as antioxidant and thus may enhance motility (Lombardo et al., 2014). Indeed, oxidative stress can have detrimental consequences for spermatozoa, such as DNA damage (Pérez-Cerezales et al., 2009) or lipid peroxidation (Shaliutina et al., 2013).

The incubation of fish sperm in buffers supplemented with antioxidants can prevent such negative effects of oxidative stress after stripping. For example, sperm treatment with 5 mM glutathione as antioxidant led to better conservation of sperm motility (57% compared to the control extender with 44%) during storage for up to 17 d at 4 °C in perch *Perca fluviatilis* (Sarosiek et al., 2014). However, VCL was lower in glutathione buffer (114 µm s^{-1}) compared to the control extender (214 µm s^{-1}; Sarosiek et al., 2014). In ram semen, a dosage of 1 mM melatonin improved progressive motility and velocity (Ashrafi et al., 2011), but the effective concentrations may differ in fish.

Here, the supplementation of KCl buffer with progesterone and melatonin were tested applying only a single concentration, since time intervals between measurements and overall sperm yield was limited. Therefore, these observations only deliver confined information on the potential of improving sperm quality after stripping and need to be further elucidated in the future.

In the second experiment, pooling effects were tested by applying a novel experimental approach of sperm and SF transfusion. An effect of transfusion could already be assessed within 15 min and was consistent for 60 min. Intra-individual transfusion did not result in alternations of sperm quality in terms of VCL, confirming the methodological approach. In only one out of 22 transfusions, it was possible to improve sperm VCL 24 h after stripping by incubation of sperm with fresh, high quality SF. In contrast, the crosswise SF transfusion experiment of males with different sperm quality (high versus low initial VCL) did not reveal positive effects. Instead, sperm quality decreased in high quality sperm after mixing with SF of low quality semen. In line with our hypothesis, it can be assumed that individual differences in SF composition substantially influence sperm quality. Therefore, pooling of sperm with varying initial quality can have detrimental consequences for the fertilization success.

Conclusion

The experiments conducted here deliver management advice for pikeperch hatchery practice. No buffer tested improved storage of freshly stripped sperm, but KCl buffer can be used to dilute sperm immediately prior to fertilization. If keeping sperm over short periods is inevitable, it should be stored untreated to minimize quality loss. In regard to buffer

supplementation, we suggest further studies across a broader range of concentrations and substances to explore the potential of antioxidants and progestins regarding the preservation and enhancement of sperm quality. Sperm pooling should only be considered after quality check. Transfusion experiments as conducted here hint at potential for enhancing or rebuilding sperm quality after storage by using SF of freshly stripped, high quality sperm.

Acknowledgements

This study was funded by the German Research Foundation (DFG KL 745/6-1). We would like to thank the staff of the project partners involved in this study. Special thanks to G. Schmidt (LFA Hohen Wangelin). We are equally grateful for the support of the AQUAGAMETE (FA1205) COST action network.

References

Alavi, S. M. H.; Cosson, J., 2005: Sperm motility in fishes. (I) Effects of temperature and pH: a review. Cell Biol. Int. 29, 101-110.

Alavi, S. M. H.; Cosson, J., 2006: Sperm motility in fishes. (II) Effects of ions and osmolality: a review. Cell Biol. Int. 30, 1-14.

Ashrafi, I.; Kohram, H.; Naijian, H.; Bahreini, M.; Poorhamdollah, M., 2011: Protective effect of melatonin on sperm motility parameters on liquid storage of ram semen at 5°C. Afr. J. Biotechnol. 10, 6670-6674.

Ashrafi, I.; Kohram, H.; Ardabili, F. F., 2013: Antioxidative effects of melatonin on kinetics; microscopic and oxidative parameters of cryopreserved bull spermatozoa. Anim. Reprod. Sci. 139, 25-30.

Bejarano, I.; Monllor, F.; Marchena, A. M.; Ortiz, A.; Lozano, G.; Jiménez, M. I.; Gaspar, P.; García, J. F.; Pariente, J.A.; Rodríguez, A. B.; Espino, J., 2014: Exogenous melatonin supplementation prevents oxidative stress-evoked DNA damage in human spermatozoa. J. Pineal Res. 57, 333-339.

Billard, R.; Cosson, M. P., 1992: Some problems related to the assessment of sperm motility in freshwater fish. J. Exp. Zool. 261, 122-131.

Blecha, M.; Křišťan, J.; Samarin, A. M.; Rodina, M.; Policar, T., 2015: Quality and quantity of pikeperch (*Sander lucioperca*) spermatozoa after varying cold water treatments. J. Appl. Ichthyol. 31, 75-78.

Bokor, Z.; Müller, T.; Bercsényi, M.; Horváth, L.; Urbányi, B.; Horváth, Á., 2007: Cryopreservation of sperm of two European percid species; the pikeperch (*Sander lucioperca*) and the Volga pikeperch (*S. volgensis*). Acta Biol. Hung. 58, 199-207.

Bokor, Z.; Horváth, Á.; Horváth, L.; Urbányi, B., 2008: Cryopreservation of pike perch sperm in hatchery conditions. Isr. J. Aquacult. Bamid. 60, 168-171.

Cabrita, E.; Sarasquete, C.; Martínez-Páramo, S.; Robles, V.; Beirão, J.; Pérez-Cerezales, S.; Herráez, M. P., 2010: Cryopreservation of fish sperm: applications and perspectives. J. Appl. Ichthyol. 26, 623-635.

Calogero, A. E.; Burrello, N.; Barone, N.; Palermo, I.; Grasso, U.; D'Agata, R., 2000: Effects of progesterone on sperm function: mechanisms of action. Hum. Reprod. 15, 28-45.

Casselman, S. J.; Schulte-Hostedde, A. I.; Montgomerie, R., 2006: Sperm quality influences male fertilization success in walleye (*Sander vitreus*). Can. J. Fish. Aquat. Sci. 63, 2119-2125.

Cejko, B.; Glogowski, J.; Kowalski, R.; Kucharczyk, D.; Targońska, K., 2008: Description of pikeperch, *Sander lucioperca* (L.), semen obtained from males held under different rearing conditions. Arch. Pol. Fish. 16, 93-100.

Cosson, J., 2004: The ionic and osmotic factors controlling motility of fish spermatozoa. Aquacult. Int. 12, 69-85.

Espino, J.; Bejarano, I.; Ortiz, Á.; Lozano, G. M.; García, J. F.; Pariente, J. A.; Rodríguez, A. B., 2010: Melatonin as a potential tool against oxidative damage and apoptosis in ejaculated human spermatozoa. Fertil. Steril. 94, 1915-1917.

Falcon, J.; Migaud, H.; Munoz-Cueto, J. A.; Carrillo, M., 2010: Current knowledge on the melatonin system in teleost fish. Gen. Comp. Endocrinol. 165, 469-482.

Gage, M. J.; Macfarlane, C. P.; Yeates, S.; Ward, R. G.; Searle, J. B.; Parker; G. A., 2004: Spermatozoal traits and sperm competition in Atlantic salmon: relative sperm velocity is the primary determinant of fertilization success. Curr. Biol. 14, 44-47.

Hanna, R.; Pang, Y.; Thomas, P.; Zhu, Y., 2006: Cell-surface expression, progestin binding, and rapid nongenomic signaling of zebrafish membrane progestin receptors α and β in transfected cells. J. Endocrinol. 190, 247-260.

Hermelink, B.; Wuertz, S.; Trubiroha, A.; Rennert, B.; Kloas, W.; Schulz; C., 2011: Influence of temperature on puberty and maturation of pikeperch, *Sander lucioperca*. Gen. Comp. Endocrinol. 172, 282-292.

Islam, M. S.; Akhter, T., 2011: Tale of fish sperm and factors affecting sperm motility: A Review. Adv. Lif. Sci. 1, 11-19.

Korbuly, B.; Grozea, A.; Cean, A.; Bănăţean-Dunea, I.; Păcală, N.; Vălean, A., 2009: Milt dilution effectiveness on pikeperch (*Sander lucioperca*) sperm DNA inactivation. Zootehnie şi Biotehnologii 42, 65-70.

Křišťan, J.; Hatef, A.; Alavi, S. M. H.; Policar, T., 2014: Sperm morphology; ultrastructure; and motility in pikeperch *Sander lucioperca* (Percidae; Teleostei) associated with various activation media. Czech. J. Anim. Sci. 59, 1-10.

Lahnsteiner, F.; Berger, B.; Weismann, T.; Patzner, R. A., 1998: Determination of semen quality of the rainbow trout, *Oncorhynchus mykiss*, by sperm motility, seminal plasma parameters, and spermatozoal metabolism. Aquacult. 163, 163-181.

Linhart, O.; Rodina, M.; Cosson, J., 2000: Cryopreservation of Sperm in Common Carp *Cyprinus carpio*: Sperm Motility and Hatching Success in Embryos. Cryobiol. 41, 241-250.

Lishko, P. V.; Botchkina, I. L.; Kirichok, Y., 2011: Progesterone activates the principal Ca^{2+} channel of human sperm. Nature 471, 387-391.

Lombardo, F.; Gioacchini, G.; Fabbrocini, A.; Candelma, M.; D'Adamo, R.; Giorgini, E.; Carnevali, O., 2014: Melatonin-mediated effects on killifish reproductive axis. Comp. Biochem. Physiol. A 172; 31-38.

Müller-Belecke, A.; Zienert, S., 2008: Out-of-season spawning of pike perch (*Sander lucioperca* L.) without the need for hormonal treatments. Aquacult. Res. 39, 1279-1285.

Murack, P. J.; Parrish, J.; Barry, T. P., 2011: Effects of progesterone on sperm motility in fathead minnow (*Pimephales promelas*). Aquat. Toxicol. 104, 121-125.

Oehninger, S.; Sueldo, C.; Lanzendorf, S.; Mahony, M.; Burkman, L. J.; Alexander, N. J.; Hodgen, G. D., 1994: A sequential analysis of the effect of progesterone on specific sperm functions crucial to fertilization in vitro in infertile patients. Hum. Reprod. 9, 1322-1327.

Pérez-Cerezales, S.; Martínez-Páramo, S.; Cabrita, E.; Martínez-Pastor, F.; De Paz, P.; Herráez, M. P., 2009: Evaluation of oxidative DNA damage promoted by storage in sperm from sex-reversed rainbow trout. Theriogenol. 71, 605-613.

Rudolfsen, G.; Figenschou, L.; Folstad, I.; Kleven, O., 2008: Sperm velocity influence paternity in the Atlantic cod (*Gadus morhua* L.). Aquacult. Res. 39, 212-216.

Rurangwa, E.; Volckaert, F. A. M.; Huyskens, G.; Kime, D. E.; Ollevier, F., 2001: Quality control of refrigerated and cryopreserved semen using computer-assisted sperm analysis (CASA), viable staining and standardized fertilization in African catfish (*Clarias gariepinus*). Theriogenol. 55, 751-769.

Rurangwa, E.; Kime, D. E.; Ollevier, F.; Nash, J. P., 2004: The measurement of sperm motility and factors affecting sperm quality in cultured fish. Aquacult. 234, 1-28.

Sarosiek, B.; Dryl, K.; Kucharczyk, D.; Żarski, D.; Kowalski, R. K., 2014: Motility parameters of perch spermatozoa (*Perca fluviatilis* L.) during short-term storage with antioxidants addition. Aquacult. Int. 22, 159-165.

Sarosiek, B.; Dryl, K.; Krejszeff, S.; Żarski, D., 2016: Characterization of pikeperch (*Sander lucioperca*) milt collected with a syringe and a catheter. Aquacult. 450, 14-16.

Scott, A. P.; Sumpter, J. P.; Stacey, N., 2010: The role of the maturation-inducing steroid, 17, 20β-dihydroxypregn-4-en-3-one, in male fishes: a review. J. Fish Biol. 76, 183-224.

Shaliutina, A.; Hulak, M.; Gazo, I.; Linhartova, P.; Linhart, O., 2013: Effect of short-term storage on quality parameters, DNA integrity, and oxidative stress in Russian (*Acipenser gueldenstaedtii*) and Siberian (*Acipenser baerii*) sturgeon sperm. Anim. Reprod. Sci. 139, 127–135.

Suquet, M.; Dreanno, C.; Fauvel, C.; Cosson, J.; Billard, R., 2000: Cryopreservation of sperm in marine fish. Aquacult. Res. 31, 231-243.

Thomas, P.; Pang, Y.; Dong, J.; Groenen, P.; Kelder, J.; De Vlieg, J.; Zhu, Y.; Tubbs, C., 2007: Steroid and G protein binding characteristics of the seatrout and human progestin membrane receptor α subtypes and their evolutionary origins. Endocrinol. 148, 705-718.

Tubbs, C.; Thomas, P., 2008: Functional characteristics of membrane progestin receptor alpha (mPRα) subtypes: a review with new data showing mPRα expression in seatrout sperm and its association with sperm motility. Steroids 73, 935-941.

Tubbs, C.; Thomas, P., 2009: Progestin signaling through an olfactory G protein and membrane progestin receptor-α in Atlantic croaker sperm: potential role in induction of sperm hypermotility. Endocrinol. 150, 473-484.

Tubbs, C.; Tan, W.; Shi, B.; Thomas, P., 2011: Identification of 17; 20β; 21-trihydroxy-4-pregnen-3-one (20β-S) receptor binding and membrane progestin receptor alpha on southern flounder sperm (*Paralichthys lethostigma*) and their likely role in 20β-S stimulation of sperm hypermotility. Gen. Comp. Endocrinol. 170, 629-639.

Uhler, M. L.; Leung, A.; Chan, S. Y.; Wang, C., 1992: Direct effects of progesterone and antiprogesterone on human sperm hyperactivated motility and acrosome reaction. Fertil. Steril. 58, 1191-1198.

Żarski, D.; Kucharczyk, D.; Targońska, K.; Palińska, K.; Kupren, K.; Fontaine, P.; Kestemont, P., 2012: A new classification of pre-ovulatory oocyte maturation stages in pikeperch, *Sander lucioperca* (L.), and its application during artificial reproduction. Aquacult. Res. 43, 713-721.

Żarski, D.; Horváth, Á.; Held, J. A.; Kucharczyk, D., 2015: Artificial Reproduction of Percid Fishes, in: Kestemont, P., Dąbrowski, K., Summerfelt, R.C. (Eds.), Biology and Culture of Percid Fishes. Springer Netherlands, Dordrecht, pp. 123–161.

Zhu, Y.; Bond, J.; Thomas, P., 2003: Identification, classification, and partial characterization of genes in humans and other vertebrates homologous to a fish membrane progestin receptor. Proc. Natl. Acad. Sci. 100, 2237-2242.

General discussion

6 Egg quality in pikeperch

Within this integrated study egg quality was assessed in commercial pikeperch broodstocks. To evaluate reproductive performance, fecundity, as well as rates of fertilization, embryo survival and hatching were determined. Several morphological, biochemical and molecular parameters of egg quality were analyzed against fecundity and developmental success of the oocytes. Furthermore, the influences of maternal traits and broodstock characteristics (out-of-season spawning) on egg composition and future oocyte development were assessed. Against the expectations, egg quality was relatively high revealing high rates of fertilization, embryo development and hatching. Still, substantial variation in reproductive success could be detected, which could partly be explained by egg quality parameters, as well as maternal characteristics. To a certain extent, suboptimal conditions at the level of female breeders were reflected in the egg composition. Consequently, multiple-regression analysis showed that embryo development could be best predicted by a combination of maternal characteristics and markers of biochemical egg composition, especially during early embryogenesis when mortality was highest. In addition, several aspects of the egg composition were strongly interlinked, such as cortisol, as well as mtDNA damage and FA profiles. Within the following sections, the findings of chapter I-III of this thesis are highlighted and jointly discussed with a focus on the mother-egg relationship.

6.1 Influence of maternal traits and out-of-season spawning

Generally, specific maternal characteristics were identified as major determinants of fecundity and egg quality in pikeperch whereas reproductive performance was largely independent of year-round reproduction suggesting no persistent perturbation of the neuroendocrine control of reproduction caused by out-of-season spawning. A schematized

overview of these findings is presented in figure 5.1 following the suggestion of Valdebenito et al. (2013) regarding the balanced and unbalanced impacts of maternal traits on egg quality.

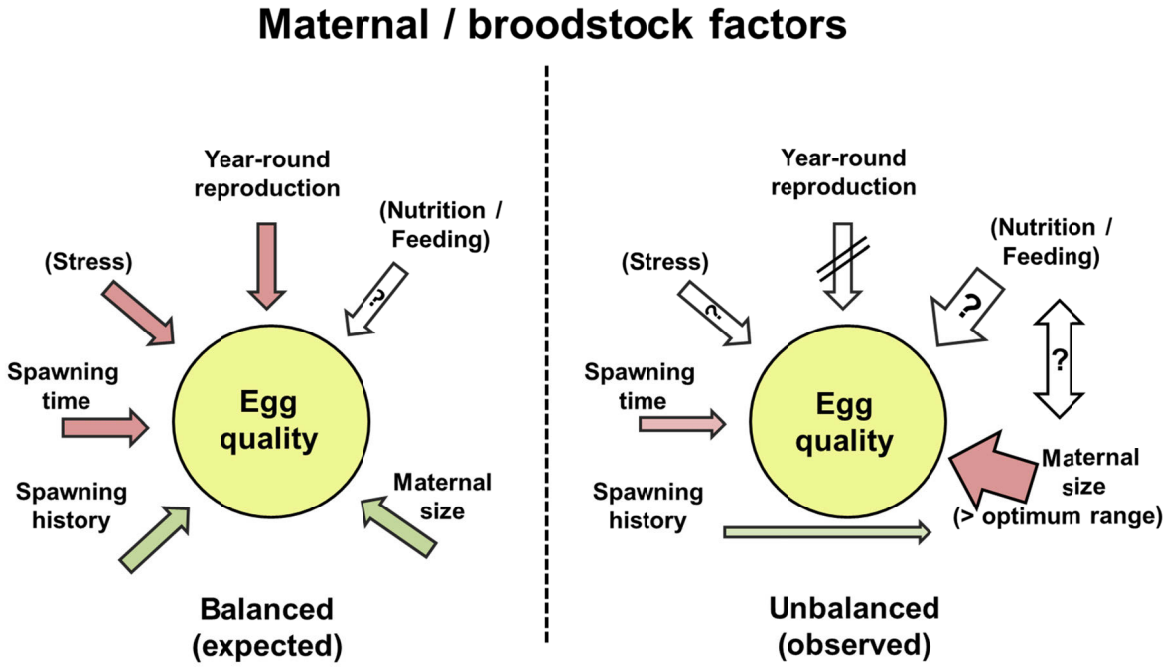


Fig. 5.1. Balanced versus unbalanced and expected versus observed effects of maternal broodstock factors on egg quality in pikeperch. Balanced factors and expected effects (green arrows = positive effect; red arrows = negative effect) are shown on the left. Unbalanced (as indicated by the size of the arrows) and observed effects are shown on the right. Crossed out arrows indicate no observed effect. Question marks indicate suggested effects as discussed within the respective chapters of this thesis. Factors set in brackets indicate effects suggested by biochemical egg composition.

Maternal length was identified as major determinant affecting fecundity, as well as the rates of fertilization and embryo survival. However, maternal length had only minor detectable influences on egg quality parameters, which in turn were correlated with egg developmental rates. Changes in egg composition, mainly FA profiles, became apparent when comparing the significantly larger successively stripped females with fish stripped for the first time. It was argued that elevated metabolic rates in larger females contributed to these observations. Therefore, size rather than spawning history explained for the increased variability in egg developmental rates of successively stripped females, which, however, were not significantly lower compared to individuals stripped for the first time. In addition, the broodstocks of the most variable spawning seasons were comprised of a mix of first-time and

successively stripped females, but fish size was not within the optimum size range of ~65 to 70 cm, which was associated with high fecundity and developmental success of the oocytes.

This could have been amplified by the handling required for *in vitro* fertilization protocols affecting the necessary recovery time in-between consecutive spawning seasons with increasing spawning history of an individual breeder. Stripping fish for *in vitro* fertilization is inevitably causing stress to the spawners. In turn, stress affects metabolic rates and exerts critical effects on reproductive mechanisms (Milla et al., 2009; Schreck et al., 2001; Strand et al., 2007). Depending on the maturation stage, stress can have significant adverse effects on female reproductive performance. For example, cortisol interferes with vitellogenesis, as well as estrogen production and indirectly disrupts the HPG axis. (cf., Milla et al., 2009 for review). However, cortisol plays a supportive role during final oocyte maturation, oocyte hydration and ovulation (Milla et al., 2006, 2009).

Pikeperch are particularly susceptible towards stress and high mortality of individuals was reported after artificial spawning, especially during year-round reproduction (Zakęś and Demska-Zakęś, 2009). In other species, it was observed that stressed females produce eggs of lower quality (Campbell et al., 1992, 1994). Furthermore, it was shown that stress-induced elevations of maternal cortisol levels are reflected in the eggs (Andersson et al., 2011; Stratholt et al., 1997) and the egg cortisol levels observed here are well in the range of previous reports on blood plasma cortisol levels of stressed female pikeperch spawners (Sarameh et al., 2012). Here, oocyte cortisol levels did not directly affect egg development, but were associated with alterations of FA profiles possibly indicating elevated metabolic demands of stressed females. Therefore, it seems not far to seek the causes of the observed increased variability in egg quality in metabolic changes of repeatedly stripped, large females caused by the procedures of artificial reproduction under hatchery conditions. However, cortisol was largely independent of female size and spawning history suggesting individual differences in stress-susceptibility.

The markers of oxidative stress did not deliver further insights regarding the adverse effects of female length. Similar to cortisol, high rates of mtDNA lesions did not directly affect egg and embryo development, but were associated with changes in the FA profiles. Mitochondrial DNA damage in the oocyte may reflect high overall oxidative stress in the entire maternal organism. Alternatively, the mother potentially incorporates higher levels of FA into oocytes as response to suboptimal conditions, such as oxidatively induced mtDNA

damage, to enable the embryo to cope with these restrains. This could further explain for the absence of negative consequences of elevated mtDNA lesions during fertilization, embryogenesis and hatching as observed here. Such maternal coping strategies have been previously suggested as protection mechanism against hypercortisolism (Schreck et al., 2001). Yet, it is not known whether the incorporation of FA into the oocyte is a response towards high mtDNA damage, which would underline the presence of a coping strategy, or if the transport of FA into the oocytes is causing oxidative stress, which in turn is reflected by mtDNA lesions. In parallel, the positive correlation of fecundity with cytb lesions could indicate higher reproductive investment in response to suboptimal conditions (coping strategy) or elevated fecundity was causing oxidative stress in the first place. Similar to the findings regarding stress-susceptibility, markers of oxidative stress did not differ in large fish or successively stripped spawners. These findings suggest individual differences, which were largely independent of other observed maternal traits.

Generally, these observations highlight the strong influence of processes on the level of the female while shaping the composition of the oocytes. However, there is a significant lack of knowledge regarding the effects of fish size and (oxidative) stress on female metabolism and subsequently, on the mobilization of nutrients and other egg components. Most certainly, potential perturbation and the mechanisms coupling individual condition, size-dependent metabolism, nutrition and the endocrine control of reproduction require further attention in future studies. Especially the leptin-kisspeptin-GnRH cascade potentially exerts an influence linking energy reserves to the neuroendocrine control of reproduction (cf., chapter I). This pathway presents a possible link between low energy reserves or high metabolic costs and decreased reproductive success. In future studies close observation of female condition, hormone levels (GnRH, LH, FSH) and steroidogenesis is suggested to further elucidate maternal processes involved in reproductive performance.

6.2 Egg quality parameters

Similar to the influence of maternal characteristics, the observed effects of quality parameters on egg development were not balanced (Fig. 5.2). In addition to egg size, several biochemical parameters could be identified as suitable biomarkers for egg developmental potential (dry weight content and absolute content of specific FA: polar 15:0, 18:0, EPA, as

well as the ratio of neutral DHA/EPA), which – to a certain extent – reflected sub-optimal conditions on the maternal level. Interestingly, no direct effect of parameters regarding oxidative stress, as well as prohibitin2 mRNA and cortisol on egg development could be observed. However, it was possible to detect links between individual egg quality parameters (e.g., FA profiles and mtDNA fragmentation, as well as cortisol), which can potentially further elucidate oocyte physiology.

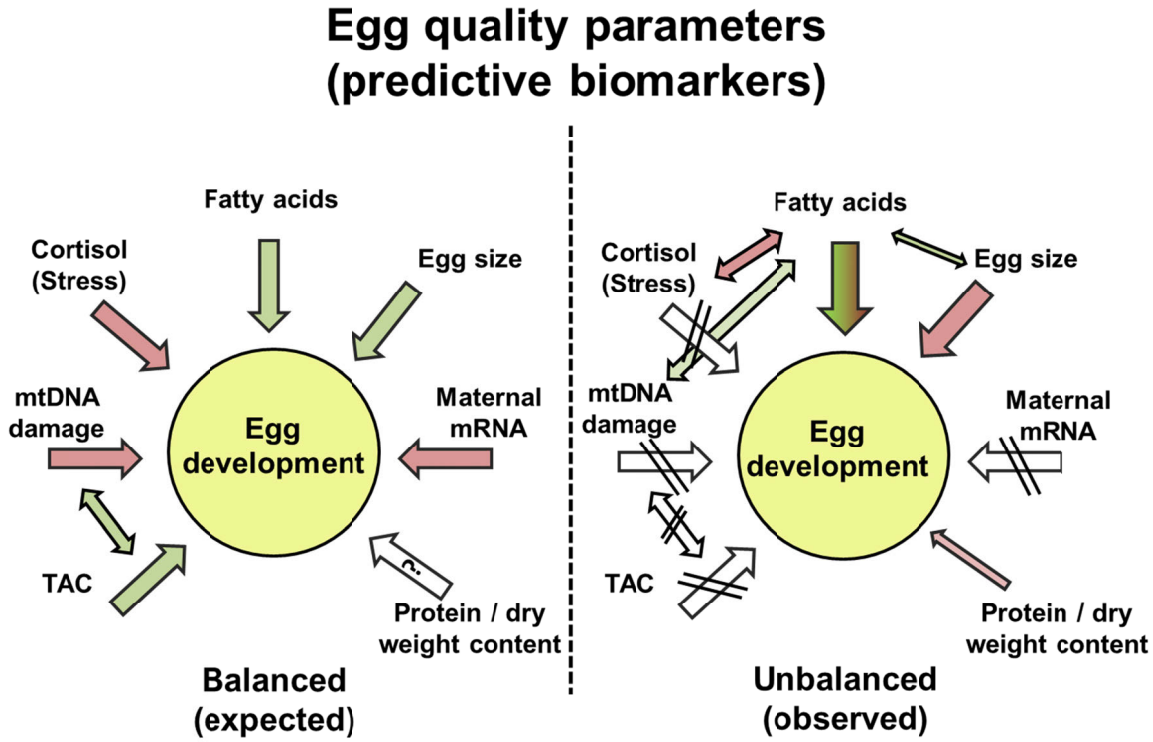


Fig. 5.2. Balanced versus unbalanced and expected versus observed effects of assessed parameters (biomarkers) on future egg development in pikeperch. Balanced and expected effects (green arrows = positive effect; red arrows = negative effect) are shown on the left. Unbalanced (as indicated by the size of the arrows) and observed effects are shown on the right. Crossed out arrows indicate no observed effect. A question mark indicates unknown expectations. In the case of fatty acids, positive (polar 15:0, EPA) and negative (polar 18:0, ration of neutral DHA/EPA) effects were observed.

Several mechanisms have been discussed partly explaining for the observed patterns in egg composition, which may be related to processes within the oocyte, e.g., the potential interactions of mitochondrial functioning and FA profiles. Yet, it remains unknown, which of these potential processes are most likely to alter egg composition since the chronology of events cannot be analyzed using the present dataset and the parameters need to be evaluated

throughout (early) ontogeny. For example, it remains unknown whether malfunctioning of mitochondria caused by mtDNA lesions is affecting the FA composition of the oocyte or vice versa. Similarly, it remains speculative, which processes are involved in regard to the observed adverse effects of egg size and dry weight content during embryogenesis.

Generally, FA profiles could be identified as major integrative determining parameters of egg quality. Specific aspects of the FA composition, such as the ratio of neutral DHA/EPA, reflected suboptimal conditions on the maternal level or could directly be used as predictive biomarkers. As discussed throughout this thesis, FA are involved in several processes, which are linked to reproduction, such as steroidogenesis and/or aspects of broodstock nutrition, as well as stress. Furthermore, there was a strong inter-linkage of FA and other egg quality parameters allowing for conclusions regarding oocyte physiology. These findings highlight the benefits of a multi-layered approach and it is thoroughly advised to take FA into account when studying egg quality.

6.3 Prediction of egg/embryo development

A substantial amount of variability in fertilization, embryo survival and hatching, as well as the number of hatched larvae could be explained by a combination of maternal characteristics and egg quality parameters using stepwise multiple regressions. Maternal size could be identified as important determinant of future egg development and successful hatching. The biochemical composition of the oocytes exerted significant effects on fertilization rate and embryo mortality during the first 24 h, especially specific polar FA (15:0, 18:0, EPA). From mid (48 h) to late (72 h) embryogenesis and during hatching, the biochemical egg composition was less suitable for predicting mortality and egg diameter, as well as total fecundity explained for the observed variability. By adding markers of oxidative stress as presented in chapter II into the regression models, no significant increase in the explained variability could be reached regarding the egg developmental potential. These results clearly demonstrate the importance of inherent composition of the oocytes especially during early embryogenesis. Here, fertilization and mortality during the first 24 h were most variable and embryos surviving until 48 h were very likely to hatch.

As indicated in chapter I, it is expected that specific egg components exert an influence on larval quality after hatching. In a previous study regarding growth of individually housed

pikeperch larvae, inter-individual differences in growth were detected under similar rearing environments (Schaefer et al., 2015). Possibly, such differences may already be ascribed to differing egg composition. Therefore, research is suggested, which extends the observed quality indicators beyond hatching as biological endpoint. However, close monitoring of external influences (temperature, external feeding and social interactions) is thoroughly advised.

It needs to be noted that the observations made here were batch-specific and not based on the composition and development of individual eggs. As discussed in chapter II, it is therefore not possible to determine whether quality parameters, such as mtDNA fragmentation, were equally distributed across batches or if individual eggs contributed disproportionally to the results representing the portion of eggs, which did not develop into a vital embryo after fertilization.

7 Sperm quality in pikeperch

Male gametes do not significantly alternate the biochemical composition of the egg. In addition, the mitochondrial genome is entirely inherited maternally. Therefore, sperm quality can mainly be described as the ability to successfully fertilize an oocyte. This ability is depending on spermatozoa characteristics, which is commonly assessed by sperm velocity and the ratio of moving to static sperm (motility). It was discussed in chapter IV that these sperm traits could directly be correlated with fertilization success in a variety of species, including the walleye, which is closely related to pikeperch. During the assessment of egg quality in chapter I and II only minimal differences in average fertilization rates could be observed in-between spawning seasons. Additionally, it was shown during the experiments dealing with sperm quality that rather inter-individual variability than reproduction protocols affected sperm quality traits. High and low quality sperm was observed independent of the applied temperature protocol or the timing of reproduction. Indeed, the sperm experiments were conducted with males, which spawned outside of the natural spawning season of pikeperch in spring. Conclusively, out-of-season spawning and the applied protocol for *in vitro* fertilization are unlikely to exert substantial adverse effects on sperm quality.

Still, it was shown in other species that paternal effects influence offspring development beyond the process of fertilization affecting early life history of the progeny. For example, it

was observed that paternity has a strong influence on hatching rate in cod (Kroll et al., 2013), as well as in haddock (*Melanogrammus aeglefinus*) (Probst et al., 2006; Rideout et al., 2004) and affected embryo development in winter flounder (*Pseudopleuronectes americanus*) (Butts and Litvak, 2007 a, b). Therefore, it can be assumed that paternal influences contributed to the observed variation in embryo development. Nevertheless, non-genetic maternal traits and egg quality parameters of unfertilized oocytes explained for significant variability in fertilization, embryo survival and hatching rate here highlighting the dominance of maternal influences.

It needs to be noted that sperm quality was visually checked prior to fertilization while assessing egg quality to prevent fertilization failure. It could be argued that this practice masked potential adverse effects of out-of-season spawning. Still, the pooling experiments of sperm and seminal fluid underlined the benefits of such a visual quality check prior to fertilization. Mixing high and low quality sperm can have a detrimental impact on sperm traits.

Under designated circumstances, e.g., in breeding programs, it may be necessary to fertilize eggs with sperm of a specific male independent of sperm characteristics regarding motility or velocity. In addition, *in vitro* fertilization protocols could greatly benefit from methods, which allow for the short-term storage of sperm to be readily available at time of ovulation. Therefore, incubation experiments were conducted to evaluate immobilization solutions for short-term storage and to identify possible supplements (melatonin, progesterone) to preserve or even to further enhance sperm quality traits. However, no beneficial effects could be detected and sperm quality decreased over time regardless of the treatment applied.

8 Implications for hatchery management

The results of this thesis deliver valuable management advice for pikeperch hatcheries and herewith support the propagation of this species in aquaculture. First, it needs to be noted that the egg quality was overall relatively high confirming the effectiveness of the applied protocol. Year-round reproduction and *in vitro* fertilization had no substantial adverse effects on egg quality. In parallel, out-of-season spawning had no detectable impact on sperm quality. Consequently, year-round reproduction in pikeperch and artificial fertilization can fully be recommended. In addition, no significant adverse effects of spawning history on reproductive

performance could be observed. However, egg quality parameters changed in response to successive stripping and cautious observation of these large females is advised especially in regard to feeding levels and resting times in-between consecutive spawning seasons. Possibly, reproduction intervals should be increased to above 12 months. Regarding the absent consequences of out-of-season spawning on reproductive performance, no adverse effects of an increased resting time are expected. Longer intervals in-between spawning seasons would potentially allow for a better recovery in response to the necessary handling.

It was indicated that parallel to increased metabolic costs of large individuals, handling stress may affect the incorporation of FA into the oocytes, which was potentially influenced by individual susceptibility. Therefore, the selection of breeders presents potential for fine-tuning of broodstock management. A female size range of ~65 to 70 cm could be identified, which was associated with high and consistent reproductive performance. It is suggested to take this under consideration for broodstock management.

To date, many pikeperch hatcheries still rely on the provisioning of forage fish (Kestemont and Henrotte, 2015; Wang et al., 2009). Feeding forage fish is a logistic burden and is further associated with the spread of diseases and parasites. Therefore, there is an urgent need for species-specific broodstock diets (Overton et al., 2015). It is recommended to formulate diets based on the findings on FA presented in this thesis.

Furthermore, the results regarding the adverse effects of larger egg diameter on egg development could only partially be explained by changes in the egg composition. It is likely that suboptimal egg incubation techniques may attribute to these observations. Small egg size may be beneficial in Zuger-jars as used here, e.g., in terms of oxygen supply or mechanical damage. Alternative incubation methods should be considered.

Regarding the management of sperm during *in vitro* fertilization it is strongly suggested to only use freshly stripped sperm. If short-term storage is inevitable, sperm should be stored at 4 °C without further treatment. Attention should be paid to sperm quality prior to pooling sperm or individual fertilization. It was shown that an initial quality check ensures high fertilization success, while pooling sperm of different males with varying initial quality can have detrimental effects.

9 Gamete quality: From pikeperch to other species?

Due to its great potential for European inland aquaculture diversification, this thesis focused on gamete quality in pikeperch. It was highlighted in several chapters that a horizontal transfer of the results presented here towards other fish species is at least difficult or may not be feasible at all. Fish represent a vast group of species with a multitude of reproductive strategies each with specific requirements for reproduction. For example, optimal oocyte FA acid composition is different in-between species or may not even be consistent within species in-between populations. Also, the effects of specific maternal mRNA on embryogenesis may not be easily transferred from one species to another as seen here. Similarly in sperm, short-term storage or enhancement supplements may have caused different effects in other fish species. Furthermore, pooling effects may differ in other species, which do not spawn pairwise, e.g., in regard to sperm competition. However, it is possible to draw some cautious generalized conclusions from the results presented here, which could be applied to other closely related percids, such as walleye, temperate species with comparable reproductive strategies or may even be valid for the majority of teleosts.

In many cases, similarities with previous reports based on observations made on other species were detected, which are not closely related to pikeperch, e.g., salmonids. For example, the interaction of mitochondria functioning and FA are likely to represent rather universally valid than species-specific processes. Furthermore, while optimal FA profiles undoubtedly vary in-between eggs of different species, the integrative role of FA as observed here represents fundamental mechanisms. Similarly, maternal influences on egg quality, such as female metabolism and/or stress, have been documented in a multitude of fish species. However, while the neuroendocrine regulation of reproduction may be similar, the environmental, social and endogenous cues triggering oocyte maturation and spawning differ strongly in-between species. Therefore, conclusions regarding the effects of year-round, out-of-season reproduction cannot be generalized, but may be valid for other temperate species.

10 Major findings

Conclusively, the major findings of this thesis are:

- No substantial adverse effects of year-round (out-of-season) spawning and applied protocols for *in vitro* fertilization on gamete quality could be observed in pikeperch.
- Maternal traits, especially female length, exerted influences on fecundity and developmental performance of the oocytes. An optimal length of ~65 to 70 cm could be identified for female spawners, which was associated with high fecundity and egg quality.
- There were indications on the maternal level regarding processes, such as handling induced and oxidative stress, successive stripping and spawning time, which affected the composition of the oocytes. Spawning history had no adverse effects on egg developmental potential.
- Inherent oocyte composition had an impact mainly during fertilization and early embryo development up to 24 h whereas egg diameter also affected late embryogenesis and hatching.
- Specific aspects of the egg composition, mainly in the context of fatty acid profiles, cortisol levels and markers of oxidative stress, were strongly interlinked suggesting oocyte based processes and/or potential coping mechanisms.
- Substantial variability in fertilization success, embryo development and the number of hatched larvae could be explained by a combination of maternal and egg characteristics.
- Loss of sperm quality during short-term storage could not be prevented using extender or enhancing supplements. Sperm should be used directly after stripping.
- The practice of pooling sperm prior to fertilization resulted in overall lowered sperm quality if males with differing initial sperm traits were used. A quality check prior to fertilization is highly recommended.

References

Alavi SMH, Cosson J (2005) Sperm motility in fishes. (I) Effects of temperature and pH: a review. Cell Biol Int 29:101-110.

Alavi SMH, Cosson J (2006) Sperm motility in fishes. (II) Effects of ions and osmolality: a review. Cell Biol Int 30:1-14.

Alavi SMH, Ciereszko A, Hatef A, Křišťan J, Dzyuba B, Boryshpolets S, Rodina M, Cosson J, Linhart O (2015) Sperm Morphology, Physiology, Motility, and Cryopreservation in Percidae. In: Kestemont P, Dabrowski K, Summerfelt RC (eds.), Biology and Culture of Percid Fishes. Dordrecht: Springer Verlag, pp. 163-191.

Almaida-Pagán PF, De Santis C, Rubio-Mejía OL, Tocher DR (2015) Dietary fatty acids affect mitochondrial phospholipid compositions and mitochondrial gene expression of rainbow trout liver at different ages. J Comp Physiol 185:73-86.

Andersson MÅ, Silva PIM, Steffensen JF, Höglund E (2011) Effects of maternal stress coping style on offspring characteristics in rainbow trout (*Oncorhynchus mykiss*). Horm Behav 60:699-705.

Bellagamba F, Caprino F, Busetto ML, Francis DS, Vasconi M, Turchini GM, Moretti VM (2011) Assessment of oxidatively generated DNA damage in rainbow trout (*Oncorhynchus mykiss*) fed with different lipid sources. Aquaculture 317:124-132.

Betancor MB, Almaida-Pagán PF, Hernández A, Tocher DR (2015) Effects of dietary fatty acids on mitochondrial phospholipid compositions, oxidative status and mitochondrial gene expression of zebrafish at different ages. Fish Physiol Biochem 41:1187-1204.

Bobe J, Labbé C (2010) Egg and sperm quality in fish. Gen Comp Endocrinol 165:535-548.

Bonnet E, Fostier A, Bobe J (2007) Microarray-based analysis of fish egg quality after natural or controlled ovulation. Bmc Genomics 8.

Brooks S, Tyler CR, Sumpter JP (1997) Quality in fish: what makes a good egg? Rev Fish Biol Fish 7:387-416.

Butts IAE, Litvak MK (2007a) Parental and stock effects on larval growth and survival to metamorphosis in winter flounder (*Pseuodpleuronectes americanus*). Aquaculture 269:339–348.

Butts IAE, Litvak MK (2007b) Parental and stock effects on embryo and early larval development of winter flounder (*Pseudopleuronectes americanus*). J Fish Biol 70:1070–1087.

Campbell PM, Pottinger TG, Sumpter JP (1992) Stress reduces the quality of gametes produced by rainbow trout. Biol Reprod 47:1140-1150.

Campbell PM, Pottinger TG, Sumpter JP (1994) Preliminary evidence that chronic confinement stress reduces the quality of gametes produced by brown and rainbow trout. Aquaculture 120:151-169.

Cerdá J, Zanuy S, Carrillo M, Ramos J, Serrano R (1995) Short-and long-term dietary effects on female sea bass (*Dicentrarchus labrax*): seasonal changes in plasma profiles of lipids and sex steroids in relation to reproduction. Comp Biochem Physiol 111:83-91.

Conover G, Simmonds R, Whalen M (eds.) (2007) Management and control plan for bighead, black, grass and silver carps in the United States. Asian Carp Working Group, Aquatic Nuisance Species Task Force, Washington D. C., 223 pp.

Cook HW, McMaster CR (2002) Fatty acid desaturation and chain elongation in eukaryotes. In: Vance DE, Vance JF (eds.), Biochemistry of Lipids, Lipoproteins and Membranes. New York: Elsevier, pp. 181-204.

Dabrowski K, Ciereszko A (2001) Ascorbic acid and reproduction in fish: endocrine regulation and gamete quality. Aquacult Res 32:623-638.

Demska-Zakęś K, Zakęś Z, Roszuk J (2005) The use of tannic acid to remove adhesiveness from pikeperch, *Sander lucioperca*, eggs. Aquacult Res 36:1458-1464.

FAO 2012. The state of world fisheries and aquaculture 2012. Rome, 209 pp.

FAO 2014. The State of World Fisheries and Aquaculture 2014. Rome, 243 pp.

FAO, 2015. FAO Global Aquaculture Production database updated to 2013 – Summary information. I4899E./1/08.15

FAO 2016. Fisheries and aquaculture information and statistics service – 09/03/2016. http://www.fao.org/fishery/statistics/en

Feiner ZS, Höök TO (2015) Environmental Biology of Percid Fishes. In: Kestemont P, Dabrowski K, Summerfelt RC (eds.), Biology and Culture of Percid Fishes. Dordrecht: Springer Verlag, pp. 61-100.

Fontaine P, Wang N, Hermelink B. (2015) Broodstock Management and Control of the Reproductive Cycle. In: Kestemont P, Dabrowski K, Summerfelt RC (eds.), Biology and Culture of Percid Fishes. Dordrecht: Springer Verlag, pp. 103-122.

Hedrick RP, Gilad O, Yun S, Spangenberg JV, Marty GD, Nordhausen RW, Kebus MJ, Bercovier H, Eldar A (2000) A herpesvirus associated with mass mortality of juvenile and adult koi, a strain of common carp. J Aquat Anim Health 12:44-57.

Hermelink B, Wuertz S, Trubiroha A, Rennert B, Kloas W, Schulz C (2011) Influence of temperature on puberty and maturation of pikeperch, *Sander lucioperca*. Gen Comp Endocrinol 172:282-292.

Hermelink B, Wuertz S, Rennert B, Kloas W, Schulz C (2013) Temperature control of pikeperch (*Sander lucioperca*) maturation in recirculating aquaculture systems-induction of puberty and course of gametogenesis. Aquaculture 400:36-45.

Izquierdo MS, Fernández-Palacios H, Tacon AGJ (2001) Effect of broodstock nutrition on reproductive performance of fish. Aquaculture 197:25-42.

Kestemont P, Henrotte E (2015) Nutritional Requirements and Feeding of Broodstock and Early Life Stages of Eurasian Perch and Pikeperch. In: Kestemont P, Dabrowski K, Summerfelt RC (eds.), Biology and Culture of Percid Fishes. Dordrecht: Springer Verlag, pp. 539-564.

Kjær MA, Todorčević M, Torstensen BE, Vegusdal A, Ruyter B (2008) Dietary n-3 HUFA affects mitochondrial fatty acid β-oxidation capacity and susceptibility to oxidative stress in Atlantic salmon. Lipids 43:813-827.

Kjørsvik E, Mangorjensen A, Holmefjord I (1990) Egg Quality in Fishes. Adv Mar Biol 1990 26:71-113.

Kroll MM, Peck MA, Butts IA, Trippel EA (2013) Paternal effects on early life history traits in Northwest Atlantic cod, *Gadus morhua*. J Appl Ichthyol 29:623-629.

Lappalainen J, Dörner H, Wysujack K (2003) Reproduction biology of pikeperch (*Sander lucioperca* (L.)) - a review. Ecol Freshw Fish 12:95-106.

Lehtonen, H., Hansson, S., Winkler, H.. (1996). Biology and exploitation of pikeperch, *Stizostedion lucioperca* (L.), in the Baltic Sea area. Ann Zool Fennici 33:525–535.

Ljunggren L (2002) Growth response of pikeperch larvae in relation to body size and zooplankton abundance. J Fish Biol 60:405-414.

McCormick MI (1998) Behaviorally induced maternal stress in a fish influences progeny quality by a hormonal mechanism. Ecology 79:1873-1883.

Mercure F, Van Der Kraak G (1996) Mechanisms of action of free arachidonic acid on ovarian steroid production in the goldfish. Gen Comp Endocrinol 102:130-140.

M'Hetli M, Ben Khemis I, Hamza N, Turki B, Turki O (2011) Allometric growth and reproductive biology traits of pikeperch *Sander lucioperca* at the southern edge of its range. J Fish Biol 78:567-579.

Migaud H, Bell G, Cabrita E, McAndrew B, Davie A, Bobe J, Herraez MP, Carrillo M (2013) Gamete quality and broodstock management in temperate fish. Rev Aquacult 5:194-223.

Milla S, Jalabert B, Rime H, Prunet P, Bobe J. (2006) Hydration of rainbow trout oocyte during meiotic maturation and *in vitro* regulation by 17,20β-dihydroxy-4-pregnen-3-one and cortisol. J Exp Biol; 209:1147–1156.

Milla S, Wang N, Mandiki SNM, Kestemont P (2009) Corticosteroids: Friends or foes of teleost fish reproduction?. Comp Biochem Physiol 153:242-251.

Monroig Ó, Rotllant J, Sánchez E, Cerdá-Reverter JM, Tocher DR (2009) Expression of long-chain polyunsaturated fatty acid (LC-PUFA) biosynthesis genes during zebrafish *Danio rerio* early embryogenesis. BBA Mol Cell Biol L 1791:1093-1101.

Morton A, Routledge R (2005) Mortality rates for juvenile pink *Oncorhynchus gorbuscha* and chum *O. keta* salmon infested with sea lice *Lepeophtheirus salmonis* in the Broughton Archipelago. Alaska Fish Res Bull 11:146-152.

Müller-Belecke A, Zienert S. (2008) Out-of-season spawning of pike perch (*Sander lucicoperca* L.) without the need for hormonal treatments. Aquacult Res 39:1279-1285.

Mylonas CC, Robles R (2014) Removing production bottlenecks of emerging species for European aquaculture. Aquaculture Europe 39:5-15.

Oakley AE, Clifton DK, Steiner RA (2009) Kisspeptin signaling in the brain. Endocr Rev 30:713-743.

Østbye TK, Kjær MA, Rørå AMB, Torstensen B, Ruyter B (2011) High n-3 HUFA levels in the diet of Atlantic salmon affect muscle and mitochondrial membrane lipids and their susceptibility to oxidative stress. Aquacult Nutr 17:177-190.

Overton JL, Toner D, Poliçar T, Kucharczyk D (2015) Commercial Production: Factors for Success and Limitations in European Percid Fish Culture. In: Kestemont P, Dabrowski K,

Summerfelt RC (eds.), Biology and Culture of Percid Fishes. Dordrecht: Springer Verlag, pp. 881-890.

Palace VP, Werner J (2006) Vitamins A and E in the maternal diet influence egg quality and early life stage development in fish: a review. Sci Mar 70:41-57.

Probst WN, Kraus G, Rideout RM, Trippel EA (2006) Parental effects on early life history traits of haddock (*Melanogrammus aeglefinus*). ICES J Mar Sci 63:224–234.

Raikova-Petrova G, Zivkov M (1998) Maturity, spawning and sex ratio of pike perch, *Stizostedion lucioperca (*L.), in two Bulgarian reservoirs as compared to other European habitats. J Appl Ichthyol 14:31–35.

Rideout RM, Trippel EA, Litvak MK (2004) Paternal effects on haddock early life history traits. J Fish Biol 64:695–701.

Rothfuss O, Gasser T, Patenge N, (2010) Analysis of differential DNA damage in the mitochondrial genome employing a semi-long run real-time PCR approach. Nucleic Acids Res 38.

Rurangwa E, Kime DE, Ollevier F, Nash JP (2004) The measurement of sperm motility and factors affecting sperm quality in cultured fish. Aquaculture 234:1-28.

Sarameh SP, Falahatkar B, Takami GA, Efatpanah I (2013) Physiological changes in male and female pikeperch *Sander lucioperca* (Linnaeus, 1758) subjected to different photoperiods and handling stress during the reproductive season. Fish Physiol Biochem 39:1253-1266.

Sargent JR, Tocher DR, Bell JG (2002) The lipids. In: Halver JE, Hardy RW (eds.), Fish Nutrition, 3rd ed. San Diego: Academic press, p. 181–257.

Schaefer FJ, Flues S, Meyer S, Peck MA (2015) Inter-and intra-individual variability in growth and food consumption in pikeperch, *Sander lucioperca* L., larvae revealed by individual rearing. Aquacul Res 1-9 (online, in press).

Schaerlinger B, Żarski D (2015) Evaluation and Improvements of Egg and Larval Quality in Percid Fishes. In: Kestemont P, Dabrowski K, Summerfelt RC (eds.), Biology and Culture of Percid Fishes. Dordrecht: Springer Verlag, pp. 193-226.

Schlumberger O, Proteau JP (1991) Production de Juvéniles de sandre (*Stizostedion lucioperca*). Aqua Revue 36:25-28.

Schlumberger O, Proteau JP (1996) Reproduction of pike-perch (*Stizostedion lucioperca*) in captivity. J Appl Ichthyol 12:149-152.

Schreck CB, Contreras-Sanchez W, Fitzpatrick MS (2001) Effects of stress on fish reproduction, gamete quality, and progeny. Aquaculture 197:3-24.

Słowińska M, Nynca J, Cejko BI, Dietrich MA, Horváth Á, Urbányi B, Kotrik I, Ciereszko A (2013) Total antioxidant capacity of fish seminal plasma. Aquaculture 400:101-104.

Steenfeldt S (2015) Culture Methods of Pikeperch Early Life Stages. In: Kestemont P, Dabrowski K, Summerfelt RC (eds.), Biology and Culture of Percid Fishes. Dordrecht: Springer Verlag, pp. 295-312.

Steffens W, Geldhauser F, Gerstner P, Hilge V (1996) German experiences in the propagation of fingerling pikeperch (*Stizostedion lucioperca*). Ann Zool Fennici 33:627-634

Stephan G, Guillaume J, Lamour F (1995) Lipid peroxidation in turbot (*Scophthalmus maximus*) tissue: effect of dietary vitamin E and dietary n-6 or n-3 polyunsaturated fatty acids. Aquaculture 130:251-268.

Strand Å, Magnhagen C, Alanärä A (2007) Effects of repeated disturbances on feed intake, growth rates and energy expenditures of juvenile perch, *Perca fluviatilis*. Aquaculture 265:163-168.

Stratholt ML, Donaldson EM, Liley NR (1997) Stress induced elevation of plasma cortisol in adult female coho salmon (*Oncorhynchus kisutch*), is reflected in egg cortisol content, but does not appear to affect early development. Aquaculture 158:141-153.

Taranger GL, Carrillo M, Schulz RW, Fontaine P, Zanuy S, Felip A, Weltzien FA, Dufour S, Karlsen Ø, Norberg B, Andersson E, Hansen T (2010) Control of puberty in farmed fish. Gen Comp Endocrinol 165: 483-515.

Teletchea F, Fontaine P (2014) Levels of domestication in fish: implications for the sustainable future of aquaculture. Fish Fish 15:181-195.

Tocher DR (2003) Metabolism and functions of lipids and fatty acids in teleost fish. Rev Fish Sci 11:107-184.

Valdebenito II, Gallegos PC, Effer BR (2015) Gamete quality in fish: evaluation parameters and determining factors. Zygote 23:177-197.

Van Densen WL, Ligtvoet W, Roozen RW (1996) Intra-cohort variation in the individual size of juvenile pikeperch, *Stizostedion lucioperca*, and perch, *Perca fluviatilis*, in relation to the size spectrum of their food items. Annal Zool Fennici 33:495-506.

Wang N, Mandiki SNM, Henrotte E, Bouyahia AG, Mairesse G, Rougeot C, Melard C, Kestemont P (2009) Effect of partial or total replacement of forage fish by a dry diet on the quality of reproduction in pikeperch, *Sander lucioperca*. Aquacult Res 40:376-383.

Wuertz S, Hermelink B, Schulz C (2012) Pike perch in recirculation aquaculture. Global Aquaculture Advocat 15:20-21.

Zakęś Z, Demska-Zakęś K (2009) Controlled reproduction of pikeperch *Sander lucioperca* (L.): a review. Arch Pol Fish 17:153-170.

Żarski D, Kucharczyk D, Targońska K, Palińska K, Kupren K, Fontaine P, Kestemont, P (2012) A new classification of pre-ovulatory oocyte maturation stages in pikeperch, *Sander lucioperca* (L.), and its application during artificial reproduction. Aquacult Res 43:713-721.

Żarski D, Horváth Á, Held JA, Kucharczyk D (2015) Artificial Reproduction of Percid Fishes. In: Kestemont P, Dabrowski K, Summerfelt RC (eds.), Biology and Culture of Percid Fishes. Dordrecht: Springer Verlag, pp. 123-162.

List of publications

Peer-reviewed articles and book chapters:

Schaefer FJ, Overton JL, Bossuyt J, Żarski D, Kloas W, Wuertz S (accepted manuscript in press) Management of pikeperch *Sander lucioperca* sperm quality after stripping. J Appl Ichthyol

Schaefer FJ, Wuertz S (2016) Insights into kisspeptin- and leptin-signalling on GnRH mRNA expression in hypothalamic organ cultures of immature pikeperch *Sander lucioperca*. Int Aquat Res (online)

Schaefer FJ, Hermelink B, Husmann P, Meeus W, Adriaen J, Wuertz S (accepted manuscript) Induction of puberty at elevated temperatures in burbot *Lota lota*. J Fish Biol

Schaefer FJ, Overton JL, Wuertz S (2016) Pikeperch *Sander lucioperca* egg quality cannot be predicted by total antioxidant capacity and mtDNA fragmentation. Anim Reprod Sci 167:117-124.

Winkelbach A, Wuertz S, Schade R, Witkowski PT, Steibli A, Meyer S, **Schaefer FJ**, Carsten Schulz (2016) Effects of oral passive immunization against somatostatin-14 on growth performance, body composition and IgY delivery in rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*). Aquacult Nutr (in press, available online)

Schaefer FJ, Flues S, Meyer S, Peck MA (2015) Inter-and intra-individual variability in growth and food consumption in pikeperch, *Sander lucioperca* L., larvae revealed by individual rearing. Aquacult Res (in press, available online)

Steinberg K, **Schaefer FJ**, Meyer S (2013) Fish feeds the world - but how?: A short review of current bottlenecks and research needs. In: Einsporn H, Wiedling J, Beilfuss S (eds.), Recent impulses to marine science and engineering. Hamburg: DGM & ICBM, pp. 99-106.

Other publications:

Schaefer FJ, Policar T, Teerlinck S, Meyer S (2015) European Percid Fish Culture (EPFC) Workshop 2014 – Summary. Aquaculture Europe 40:33-36.

Schaefer FJ, Kloas W, Würtz S (2012) Arapaima: Candidate for Instensive Freshwater Culture. Global Aquaculture Advocat 15:50-51.

Eidesstattliche Erklärung

Ich erkläre hiermit, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Literatur und Hilfsmittel angefertigt habe. Beim Erstellen dieser Dissertation bestand keine Zusammenarbeit mit gewerblichen Promotionsberatern. Ich habe die dem angestrebten Verfahren zur Grunde liegende Promotionsordnung zur Kenntnis genommen und habe die Dissertation nicht bereits bei einer anderen wissenschaftlichen Einrichtung ganz oder in Teilen eingereicht. Die Grundsätze der Humboldt-Universität zur Sicherung guter wissenschaftlicher Praxis wurden eingehalten. Ich erkläre hiermit, dass ich zuvor noch keinen Promotionsantrag gestellt habe bzw. einen entsprechenden Doktorgrad besitze.

Berlin, 08.06.2016

_____

Fabian J. Schäfer